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OPTIMIZED BIOPROCESSING OF WHEAT BRAN FOR MAXIMAL PROTEIN SOLUBILIZATION

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Tiivistelmä – Referat – Abstract

The aim of this thesis was to create an optimized bioprocessing to solubilize the maximal amount of proteins from the wheat bran without losing their nutritional and technological quality. The hypothesis of this thesis was that a maximal degradation of the aleurone cell wall components would lead to a maximal amount of soluble proteins originally located inside the aleurone layer. The literature review further looked into possible extraction methods for wheat bran proteins.

Fine wheat bran was chosen to be bioprocessed by using experimental design to find optimal conditions for protein solubilization. Bioprocessing was done either by using starter culture alone or a combination of selected enzymes and starter culture. Independent studied factors were time (8 h, 16 h, 24 h), temperature (20 °C, 27.5 °C, 35 °C) and enzyme dosage (5 nkat/g, 50 nkat/g, 500 nkat/g). Optimization was carried out by applying response surface methodology to analyze the relationship between the response and the independent variables.

Optimized bioprocessing of wheat bran led to maximal protein solubilization of >50% for wheat bran both bioprocessed with starter culture and with starter culture and enzymes after fermentation time of 24 h. Thus, the amount of soluble protein increased 23%. This indicated that the use of enzymes did not improve the breaking down of the aleurone cell walls for protein liberation. Furthermore, the use of enzymes affected heavily the protein degradation for fermentations longer than 8 h. Since the amount of solubilized protein was higher for wheat bran bioprocessed for 8 h with starter only (>46%) than for wheat bran bioprocessed for 8 h with starter and enzymes (>40%), the use of enzymes for a larger scale production does not seem feasible.

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Tiivistelmä – Referat – Abstract

Tutkimuksen tavoitteena oli luoda optimoitu bioprosessointi, jonka avulla vehnäleseen proteiineista saataisiin suurin mahdollinen osa liuotettua. Samalla kiinnitettiin huomiota siihen, että proteiinien teknologinen ja ravitsemuksellinen laatu ei häviäisi. Tutkimuksen hypoteesin mukaan aleuronikerroksen soluseinien maksimaalinen hajottaminen johtaisi maksimaaliseen määrään liuotettua proteiinia. Tutkielman kirjallisuuskatsauksessa perehdyttiin myös laajemmin vehnäleseen proteiinien uuttomenetelmiin.

Hienoksi jauhettu vehnälese valittiin bioprosessoitavaksi. Bioprosessoinnissa käytettiin tilastollista koesuunnittelua määriteltäessä optimaalisia olosuhteita proteiinien liukoisuudelle. Bioprosessoinnissa käytettiin joko kaupallista maitohappobakteeriviljelmää, tai kaupallisen maitohappobakteeriviljelmän ja kaupallisten entsyymien sekoitusta. Tutkimuksessa seurattiin kolmea toisistaan riippumatonta muuttujaa: aika (8 h, 16 h, 24 h), lämpötila (20 °C, 27.5 °C, 35 °C) ja entsyymien annostus (5 nkat/g, 50 nkat/g, 500 nkat/g). Optimoinnissa käytettiin vastepintamallitusta, jonka avulla analysoitiin vasteiden ja riippumattomien muuttujien välisiä suhteita.

Optimoidun bioprosessoinnin tuloksena vehnäleseen liukoisten proteiinien määräksi saatiin >50%. Tämä tulos saatiin näytteille, joiden bioprosessoinnissa käytettiin joko kaupallista maitohappobakteeriviljelmää, tai kaupallisen maitohappobakteeriviljelmän ja kaupallisten entsyymien sekoitusta, ja joiden fermentoitumisaika oli 24 h. Näin ollen liukoisen proteiinin määrä lisääntyi 23%. Entsyymien käyttö ei edistänyt aleuronikerroksen soluseinien hajottamista. Entsyymien käyttö vaikutti voimakkaasti proteiinien hajoamiseen näytteissä, joiden fermentoitumisaika oli pidempi kuin 8 h. Liukoisen proteiinin määrä oli suurempi 8 h ajan vain maitohappobakteeriviljelmällä fermentoidussa näytteessä (>46%) kuin maitohappobakteeriviljelmällä ja entsyymeillä fermentoidussa näytteessä (>40%). Näin ollen entsyymien käytössä laajamittaisessa tuotannossa ei ole hyötyä.

Avainsanat – Nyckelord – Keywords
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PREFACE

This Master´s thesis was done at the Department of Food and Nutrition at the University of Helsinki. The thesis was part of the Lantmännen-funded Biobran-project between the Grain technology group and Lantmännen. This thesis was supervised by Associate Professor Kati Katina and PhD candidate Elisa Arte.

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1 INTRODUCTION

There is a rising need for plant-based protein, which can be used to create new food applications or to fortify existing food products by supplying protein. This need is driven by the rise of the world population, and the inefficient use of plant based proteins as feed by the meat industry. Today, wheat is the world´s most important protein source, and assumed that all of the wheat assigned for human consumption is milled, it would generate a by-product stream of 150 million tons of wheat bran on a yearly basis (Shiferaw et al. 2013, Prückler et al. 2014). Currently wheat bran is used mostly as feed for livestock.

Wheat bran is the outermost part of the wheat grain and it is known as a good source of dietary fibre, vitamins and minerals. (Kamal-Eldin et al. 2009). Lately more emphasis has been shown towards the proteins of the wheat bran (Arte et al. 2015 and 2016, Balandran-Quintana et al. 2015, De Brier et al. 2015). The protein content of the wheat bran is 16–20%. (Poutanen et al. 2012, Rizzello et al. 2012, Coda et al. 2014). Most of the proteins of the wheat bran are located in the aleurone layer, which is botanically seen as the outer layer of the endosperm, but is separated from the endosperm during milling, and hence regarded as a part of the bran by cereal scientists (Brouns et al. 2012). Wheat bran proteins consist mostly of albumins and globulins (De Brier et al. 2015, Idris et al. 2003), and they differ in their biological function and location in the bran. The aleurone layer has approximately 15% of the proteins of the wheat kernel. The most abundant proteins in the aleurone layer are globulin-like storage proteins.
In human nutrition, wheat bran is mostly used in baking and cereal products as a dietary fibre component.

Effective extraction of wheat bran proteins is difficult, because the majority of the proteins are entrapped in the aleurone cells (Brouns et al. 2012). Aleurone cell walls consist of insoluble fibre and structural proteins, which do not release the proteins to human digestion. The solubility of wheat bran proteins can be improved by bioprocessing with selected starter cultures and commercial enzymes (Arte et al. 2015 and 2016, Coda et al. 2014). Bioprocessing of wheat bran also improves nutritional quality of the wheat bran by increasing the amount of essential amino acids, the in vitro digestibility of proteins and bioavailability of phenolic compounds (Arte et al. 2015). Bioprocessing affects the proteins and fibres of wheat bran in several ways. During fermentation the pH drops as a result of

lactic acid bacteria metabolism (Loponen et al. 2004, Katina et al. 2012), and this decline in the pH activates both endogenous and bacterial enzymes, which have proteolytic and carbohydrolytic activities and affect the structures of wheat bran cell walls and proteins. Bioprocessing is a selection of techniques, where selected micro-organisms and other exogenous enzymes are used under controlled circumstances (temperature, time) to obtain desirable changes in in the food matrix. Bioprocessing was used in this work as a means to break down the cell walls of the aleurone cells, and to improve the bioaccessibility and bioavailability of wheat bran proteins and other compounds.

Experimental design is a tool to conduct and plan experiments to obtain the maximum amount of information from the collected data by conducting the minimum of experiments. Experimental design is widely used when aiming for industrial scale processes. Response surface methodology (RSM) is an experimental methodology that analyses the relationship between the response and the independent variables (Bas et al. 2007). RSM has been used in previous research to study optimized sourdough process conditions and the effects of fermentation on the solubility of pentosans in rye bran (Katina et al. 2005 and 2006, Hartikainen, K. 2011).

The aim of the study was to create an optimized bioprocess and solubilize the maximal amount of proteins, without losing their nutritional and technological quality, from the wheat bran. The process should be applicable also on an industrial level.

2 LITERATURE REVIEW

2.1 Wheat bran proteins

2.1.1 Protein content and quality

The wheat bran consist of three layers, which can be mechanically separated (Antoine et al. 2003, 2004). According to the botanical definition, the wheat bran consists of two layers – the testa and the pericarp, which means that botanically observed the aleurone layer is the outermost part of the endosperm. In a technological point of view the bran consist of the three layers mentioned above. This is due to the aim of milling where the goal is to separate the starchy endosperm as effectively as possible to obtain a white clean flour.

The outermost part of wheat bran is the pericarp, and it consists mostly of insoluble fibre and bioactive compounds (Surget and Barron 2005, Brouns et al. 2012). The middle layer is the testa, and the innermost layer of the bran is the aleurone layer. The aleurone layer consist of living aleurone cells. In the aleurone layer, the storage proteins are located in protein storage vacuoles inside the aleurone cells (Bethke et al. 1998). Structural proteins are located in the cell walls of the aleurone cells (Rhodes and Stone 2002). The protein content of different wheat bran layers was studied by Jerkovic et al. (2010), where microdissection of the wheat bran layers was used to obtain information of the wheat bran proteins. The pericarp has the least protein content (0.4 mg/g). The protein content in the testa is 3.6 mg/g, and the protein content of the aleurone layer is 156 mg/g. This corresponds to a total protein content of the wheat bran of 19.6%. Similar protein contents for the wheat bran ranging from 16 to 20% have been presented (Poutanen et al. 2012; Rizzello et al. 2012). Nevertheless, Brouns et al. (2012) presented a significantly higher protein content for the aleurone layer, which is 30%.

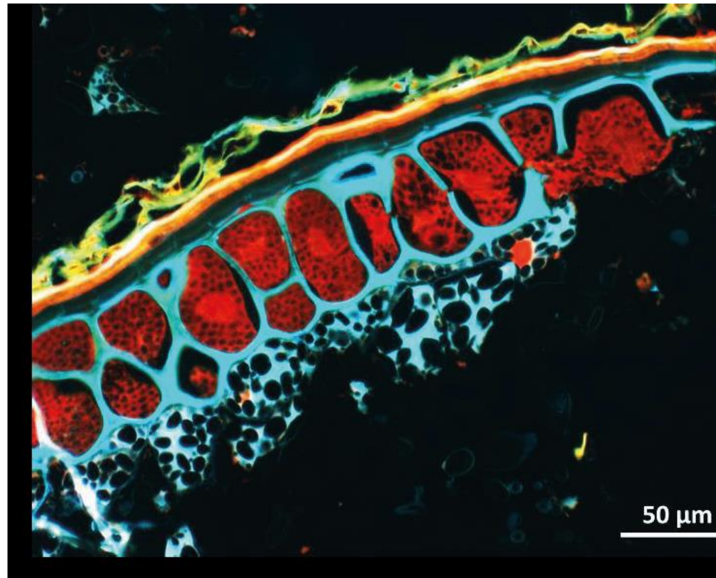

Figure 1. Micrograph of wheat bran. The bran structures from the outside to the inside are: pericarp layer in light green/yellow, testa and hyaline layer in orange and cell walls of aleurone layer and endosperm as light blue. The proteins appear as red. The bran was stained with Acid Fuchsin and Calcofluor white. Image courtesy of VTT Ltd / Ulla Holopainen-Mantila.

According to Jerkovic et al. (2010), the bran proteins are uneven distributed because each layer has its own biological function. Meziani et al. (2012) observed that the protein content of wheat and wheat bran varies between cultivars. The protein content is also dependent from different growth factors as weather, available nutrients and pathogens.

Wheat proteins are often classified according to their solubility. This classification is based on the work conducted by T.B. Osborne. According to Osbornes classification (Osborne 1907), albumins are water-soluble, and these proteins are also coagulated by heat. Globulins are soluble in dilute salt solutions, and gliadins are soluble in aqueous 70% ethanol, whereas glutenins are soluble in dilute acids or bases. According to Belderok et al. (2000) albumins and globulins are small proteins, albumins being the smaller ones. Most of the proteins in wheat are albumins and globulins, and they are located in the aleurone layer and in the germ (Bethke et al. 1998). Gliadins and glutenins are larger proteins and have more complex structures, and these proteins are storage proteins that are located mostly in the endosperm, as stated by Belderok et al. (2000). Glutenins and gliadins form together gluten, which gives wheat its unique baking properties.

Wheat bran proteins were determined according to the Osborne classification by De Brier et al. (2015). The results indicated that most of the proteins in wheat bran belong to the albumin/globulin fraction (33%), followed by glutelins (16%) and gliadins (11%). Accordingly, 40% of proteins were insoluble.

In another classification of wheat proteins the proteins are divided into superfamilies. A protein superfamily is defined by Barker et al. (1978) to consist of related proteins whose sequence homology is limited. According to their study, two known superfamilies of wheat proteins are the prolamin superfamily and the cupin superfamily. According to Kreis et al. (1985) the prolamins consist of three groups of seed proteins that have a similar model of cysteine residues. These are the sulfur-rich prolamins of the *Triticeae*, the cereal α-amylase/trypsin inhibitors and the 2S storage albumins of oilseed rape, castor bean and other dicotyledonous seeds. As further stated by Kreis et al. (1985), the cupins are present in many organisms as various bacteria and plants. For cupins, the similarity between the proteins is obvious when their three-dimensional structures are compared. In this case, the pattern is a β-barrel shape. According to Woo et al. (2002) some of the best known cupins are oxalate oxidase, auxin-binding protein 1 and the 7S and 11S storage globulins of legumes and cereals.

2.1.2 Proteins of the aleurone layer

The aleurone layer makes up approximately 50% of the bran fraction (Brouns et al. 2012). The layer consists of one cell layer, and it contains most of the brans minerals, vitamins, phenolic antioxidants and lignans (Brouns et al. 2012, Buri et al. 2004). The protein content of the aleurone layer is fairly high, 15–30%, and the aleurone layer contains 15% of the proteins of the kernel (Idris et al. 2003, Jerkovic et al. 2010, Brouns et al. 2012). According to Buri et al. (2004), there are high levels of essential amino acids such as lysine, threonine and valine in the aleurone layer, whereas contents of sulfur-rich amino acids methionine and cysteine are low.

According to Jerkovich et al. (2010) the proteins of the aleurone layer have broader functionality than the proteins from the testa and pericarp. The main function of the aleurone cells is to provide organic substrates to the endosperm during germination (Bethke et al. 1998). As presented in the studies by Jerkovic et al. (2010) and Bethke et al. (1998), most of the proteins of the aleurone layer are globulin-like storage proteins, whereas other major proteins consist of different dehydrogenase enzymes. The dehydrogenase enzymes participate in protein synthesis, carbohydrate metabolism and stress and defense reactions of the cell layer. In a study by Rizzello et al. (2012) wheat bran was micronized and air fractioned to obtain various fractions that can be used in bread baking. The amount of

proteins according to the Osborne classification in the fraction consisting of aleurone cells and the innermost layers of the aluerone layer was defined to be as follows – albumins and globulins 61.8%, gliadins 24.4% and glutenins 13.8%. In this case the amount of proteins was calculated from the total amount of fractionated proteins.

The protein content of the aleurone layer of two different wheat cultivars can vary significantly. The proteins of the aleurone layer of the two main wheat cultivars *Triticum aestivum* and *Triticum durum* were examined in a study by Meziani et al. (2012). According to the findings most of the proteins were globulins. Interestingly, the quantities of the proteins varied, and the aleurone layer of *Triticum aestivum* was to found to have 10% more globulins than *Triticum durum*. This difference was explained to be due to differences in the genomes of the two cultivars.

In a study by Rhodes and Stone (2002), the proteins of the aleurone cell walls were classified as hydroxyproline-rich glycoproteins, glycine-rich proteins and proline-rich proteins according to their solubility in different extraction media. Furthermore they conducted that these proteins serve as structural proteins, since they form very resistant cross-links with one another and other cell wall polymers. This makes the extraction of cell wall proteins challenging, and it complicates the access to the nutritionally valuable contents inside the cell, as concluded by Rhodes and Stone (2002), whereas with genetic manipulation, it would be possible to control the synthesis of aleurone cell walls and in this way get a cell wall which would be more breakable during milling.

2.1.3 Proteins of the pericarp and the testa

The main purpose of the proteins of the pericarp is to protect the seed against external biological and chemical effects, as pointed out in the comprehensive study by Jerkovic et al. (2010). They concluded that these proteins consist mostly of oxidative stress- and defense-related proteins, such as oxalate oxidase, lipoxygenase and lipid transfer protein. According to Jerkovic et al. (2010), the proteins of the testa consist as well of oxidative stress- and defense-related proteins, but the diversity of the proteins is much broader, and many inhibitor proteins are located in this layer.

Furthermore, plants contain endogenous inhibitor proteins whose purpose it is to reduce the activity of endo- and exogenous enzymes (Juge and Svensson 2006). According to their study, there are many inhibitor proteins in cereals, whose targets are the starch degrading enzymes. Inhibitor proteins in wheat are α-amylase inhibitors and xylanase inhibitors, of which both are located in the testa and aleurone layers of the bran, as stated in the study mentioned above. Amylases degrade (1→4)-α-D-links of the glucose polymer, and amylase inhibitors hinder the degrading work of α-amylase, whereas xylanases hydrolyse (1→4)-β-links and degrade arabinoxylan (AX) (MacGregor et al. 2001). According to Juge and Svensson (2006), three different types of xylanase inhibitors were found out to be present in wheat bran. They are *Triticum aestivum* xylanase inhibitor, xylanase inhibiting protein and thaumatin-like xylanase inhibitor. Xylanase inhibitors regulate the activity of xylanases, and indirectly affect the viscosity and the water holding capacity of doughs, and in baking processes xylanases have have a big impact on dough properties, like softness, stability and elasticity of the dough (Gys et al. 2003). Furthermore, the presence of α-amylase inhibitors affects baking and brewing processes through regulation of the activity of amylase, as stated by Munck et al. (1985).

2.2 Bioprocessing

Bioprocessing with baker´s yeast and lactic acid bacteria fermentation and/or hydrolyzing enzymes has been previously used to enhance the flavor and loaf volume of breads baked with wheat bran (Salmenkallio-Marttila et al. 2001, Katina et al. 2012, Coda et al. 2014). Bioprocessing has in addition been used to promote the bioaccessibility of nutritionally beneficial compounds in bran (Coda et al. 2014, Katina et al. 2007, Anson et al. 2009). Wheat bran bioprocessing with enzymes and/or lactic acid bacteria and yeast has also been shown to increase the content of bioactive compounds in bread with possible positive physiological effects (Anson et al. 2011).

In fermentation, micro-organisms such as lactic acid bacteria (LAB) oxidize glucose as part of their metabolism, and create end products that are acids, alcohol and carbon dioxide. There are several fermentation processes, which can be recognized by the created end products (Mehta et al. 2012, Corsetti and Settanni 2007). Ethanol and carbon dioxide are the end products of ethanol fermentation, and this fermentation process occurs in yeasts and to some content in bacteria, as stated by Corsetti and Settanni (2007). As pointed out in their study, the lactic acid fermentation is caused by LAB, and is divided into two pathways

according to their end products, which are homolactic fermentation that results to lactic acid, and heterolactic fermentation that results to equal amounts of lactic acid, ethanol and carbon dioxide.

Fermentation can begin spontaneously, if the growing conditions, as temperature and pH, are beneficial for the endogenous micro-organisms of the food matrix, or if exogenous micro-organisms are added from the environment (Mehta et al. 2012, Corsetti and Settanni 2007). As observed by Mehta et al. (2012), in industrial food processing controlled circumstances for fermentation are required to achieve microbiologically safe products of consistent quality, and to avoid food waste caused by spoilage bacteria. Therefore, industrial fermentation of cereals occurs as a combination of selected strains and the indigenous microbiota naturally present in grains.

2.2.1 Effects of bioprocessing on wheat bran proteins

Bioprocessing affects the proteins of wheat bran in several ways. During fermentation the pH declines due to LAB metabolism (Loponen et al. 2004, Khan and Shewry 2009, Katina et al. 2012), and this drop in the pH activates both endogenous and bacterial enzymes. Both endogenous and microbial originated enzymes are concentrated on the outer layers of the grain (Dornez et al. 2006, Gys et al. 2004), and these enzymes will be activated during the bran fermentation. The final pH value after fermentation depends on the time and temperature of the fermentation, and the amount and quality of the strains added (Loponen et al. 2004, Gänzle et al. 2008). Proteolytic enzymes, or proteases are grouped into proteinases and peptidases Gänzle (2014). Proteinases catalyze protein degradation into smaller peptide fractions, where peptidases hydrolyse specific peptide bonds or completely break down peptides to amino acids (Thiele et al. 2004, Gänzle et al. 2008). As pointed out by Gänzle et al. (2008) proteolysis can be divided to primary and secondary proteolysis, where primary proteolysis degrades proteins to peptides and secondary proteolysis further degrades peptides to amino acids. In their study, it is observed that primary proteolysis is dependent on endogenous cereal proteases.

Degradation and depolymerization of proteins affects the functionality and the nutritional value of these components (Gänzle 2014, Gänzle et al. 2007). With bioprocessing, it is possible to influence on the amount and quality of the resulting peptides and amino acids.

According to Gänzle et al. (2008), the proteases present in the endosperm of wheat grain are aspartic proteinases and serine carboxypeptidase II. The pH range for aspartic proteases is 3–4.5, and for serine carboxypeptidase II 4–6. According to Breddam et al. (1987) the endogenous proteolytic activity of the wheat bran is mainly due to serine carboxypeptidases. As presented in their study, the optimum pH level for these enzymes is 4–4.5. According to Gänzle (2008), the degradation and depolymerization of proteins during sourdough fermentation is dependent on bacterial metabolic activity and cereal enzymes.

Arte et al. (2015 and 2016) have previously studied the use of different bioprocessing methods to examine the release and modification of the wheat bran proteins. Different combinations of exogenous enzymes and selected strains were used to solubilize and degrade wheat bran proteins. This study highlighted the problem that since most of the wheat bran proteins are located inside the aleurone cells, it is necessary to break down the fibrous cell walls in order to liberate the proteins. If the aleurone cell walls are not broken down, the proteases can only degrade proteins that are on the outside of the aleurone cell, as stated by Arte et al. (2016). As concluded in the study, the desired end products after proteolysis are peptides with an increased digestibility and beneficial bioactive functions. A balanced outcome of peptides and amino acids is crucial to obtain their beneficial characteristics as taste and flavor precursors and bioactive compounds (Gänzle 2014). Too intensive proteolysis results in high amounts of free amino nitrogen with low nutritional value due to lower protein digestibility (Coda et al. 2013).

Fermentation of wheat bran has been used previously in baking, where fermented wheat bran has shown several positive effects concerning technical and nutritional aspects (Poutanen et al. 2009, Rizzello et al. 2010 and 2012). During fermentation in sourdough bread baking proteins are degraded and accordingly they account to the overall quality such as flavor, volume and texture of sourdough bread, as stated by Gänzle et al. (2007) and Katina et al. (2007). Amino acids and peptides are precursors for volatile flavor compounds and affect the taste of fermented foods (Coda et al. 2012, Gänzle 2014). The metabolism of sourdough microbiota and the activity of cereal enzymes are interdependent, and acidification modifies the activity of cereal enzymes and the solubility of substrates, such as gluten proteins and phytate (De Vuyst and Neysens, 2005). According to Bleukx et al., (1998) and Brijs et al. (1999) sourdough fermentation shifts the ambient pH to the optimum pH of aspartic proteases, the major proteinase in resting grains of wheat and rye. According to Gänzle et al. (2008) proteolysis in wheat and rye sourdough remains limited to degradation of less than

5% of the cereal proteins, therefore extensive protein degradation requires addition of malt or fungal enzymes.

2.2.2 Effects of bioprocessing on wheat bran fibres

The fibre of wheat bran is mainly composed of the cell wall polysaccharides AX (64%), cellulose (29%), and non-cellulosic glucan (6%) (Fincher & Stone, 1986). According to Izydorczyk and Biliaderis (1992), the structure of these polysaccharides is cross-linked by small phenolic acids, such as ferulic acid (FA). A high degree of cross-linking increases the molecular size of the polysaccharides and reduces their solubility. The aleurone cell wall consists of 29% β-glucans, few proteins, and 65% relatively linear AX with a low arabinose to xylose ratio (A/X), and high amounts of esterified FA monomers (Bacic and Stone 1981, Rhodes et al. 2002, Saulnier et al. 2007). This natural composition of the bran and the aleurone cell walls complicates the bioaccessibility of the wheat bran proteins, since the nutritionally most valuable proteins are located inside the aleurone cell walls, as stated by the studies mentioned above. The desired outcome of wheat bran bioprocessing is to degrade resistant fibres so that access to the aleurone cells can be established.

As stated by Izydorczyk and Biliaderis (1992), the insolubility of cell walls can partly be explained by the presence of phenolic compounds. Especially FA´s are part of the cross-links which form between lignin and AX polysaccharides (Faulds and Williamson 1999). AX consist of a linear β-(1.4) linked xylan backbone to which α-L-arabinofuranose units are attached as side residues via α-(1.3) and/or α-(1.2) linkages (Andrewartha et al. 1979). As pointed out in their study, FA or diferulate residues are esterified to arabinose residues at O-5. In a study by Katina et al. (2012) impacts of fermentation type and type of wheat bran on the microbial community, activity of xylanases and AX were investigated. If bran was fermented at 20 °C with a modest LAB growth and only slight acidification (pH level of 6–6.5), the highest level of free FA was found. According to Boskov Hansen et al. (2002), this pH level is close to the optimum pH (7) of cinnamoyl esterases found in whole grain flour. In their study, the lowest level of free ferulic acid was detected in a strongly acidic (pH 3.9–4.1) environment, and supposedly, this strong acidicity inhibits the cinnamoyl esterase. The same trend with higher ferulic acid levels was clear in all other fermentations that had higher pH values. Hence, the amount of free FA can be used as an indicator of the breakdown of AX.

According to Boskov Hansen et al. (2002) the solubilization of AX during the baking of whole-meal rye was due to the activity of xylan-degrading enzymes and acidification of the matrix. A decrease of pH due to LAB fermentation leads to the activation of endogenous degrading enzymes such as amylase, pentosanase, and β-glucanase (Dornez et al. 2006, Gys et al. 2004). The enzymes hydrolyze to some extent dietary fibres to sugars, which become available for fermentation by the lactic acid bacteria (Katina et al. 2005). Endogenous xylanase activity in native bran was to be found higher compared to the peeled bran. This indicates that microbial xylanases are only or almost entirely located in the outer layers of grain (Gebruers et al. 2008, Dornez et al. 2009). Thus, debranning greatly reduces endogenous xylanase activity (Gys et al. 2004). Furthermore, xylanase inhibitors from bran can inhibit microbial xylanases, which are probably partly responsible for the higher endogenous activity of native bran.

The effect of hydrolyzing enzymes on wheat bran cell wall integrity was studied by Arte et al. (2016). The exogenous enzymes used in their study were commercial enzymes, which consisted of mixtures of carbohydrate-hydrolyzing and proteolytic enzymes. The degradation of cell walls was analyzed by determining the content of water-extractable (WE) pentosan and reducing sugars. As pointed out in their study, the content of WE pentosan represents hydrolysis products such as AX and arabinogalactans, whereas the content of reducing sugars represents hydrolysis products as β-glucan and cellulose. It was observed that the WE pentosan content increased significantly in samples with addition of Depol 761P (high xylanase activity), Viscoferm (high xylanase and β-glucanase activity) and Viscozyme L (high polygalacturonase activity and endoglucanase activity, with proteolytic side activity) at all incubation times (4, 6 and 16 h). On the other hand, the other carbohydrate-hydrolyzing enzymes, Econase CE (high xylanase and β-glucanase activity), Celluclast 1.5 L (high β-glucanase and endoglucanase activity), and Glucanase 5XL (β-glucanase) led to smaller increases in WE pentosan content after incubation time of 4 and 6 h. This might be explained by the metabolic activity of endogenous enzyme inhibitors, and selectivity of the exogenous enzymes towards soluble or insoluble arabinoxylans (Berrin and Juge 2008). The WE pentosan content did not increase after incubation time of 16 h. According to Arte et al. (2016), the same trend in the WE pentosan content, increasing for 4 and 6 h incubation and decreasing for 16 h incubation, was to be found for most of the tested enzymes. A further explanation as stated by Gänzle et al. (2007) can be the metabolic activity of the endogenous lactic acid bacteria of the bran, in which the hydrolyzed carbohydrates are consumed by lactic acid bacteria. The same trend was obvious also for the reducing sugar content in the

study by Arte et al. (2016). And again, the decrease of the reducing sugar content after longer incubation time can be explained by metabolic activity of LAB and yeasts, as stated by Gobbetti and Gänzle (2013). According to Arte et al. (2016) the most effective enzymes to increase the WE pentosan and reducing sugar contents were Depol 761P and Viscozyme L.

Further experiments on enzymatic and chemical degradation of polysaccharides in wheat aleurone cell wall have been conducted by Rhodes and Stone (2002). The aim of their research was to gather more information on the polysaccharides and proteins of the aleurone cell wall. Since their goal was not to liberate the proteins inside the aleurone cell for food grade use, their approach was also somewhat different. The cell walls were extracted with apriotic solvents such as dimethylacetamide-lithium chloride and dimethylsulfoxide-paraformaldehyde and hot water. After extraction, the cell walls were incubated with polysaccharide hydrolases (glucanase and xylanase). Even after quite extensive treatments, the aleurone cell wall could not be solubilized completely. According to Bacic and Stone (1981), most of the polysaccharides in the aleurone cell wall can be removed by water at 40 °C and 8 M urea, whereas the remaining part of the cell wall consists of structural proteins with covalent cross-linkages to the matrix polysaccharides.

According to Coda et al (2012), enzyme addition during bran fermentation improved bread texture and resulted in enhanced softness of both fresh and stored breads in comparison to control wheat bread and with the control breads enriched with bran.

It should be noted, that the particle size of wheat bran has an effect on the solubilization of wheat bran fibres, as stated by Coda et al. (2014). In their study, the solubility of AX increased by fractionation. The highest content of solubilized AX was in the finest bran (50 µm).

2.2.3 Nutritional effects of bioprocessing

The abundant presence of dietary fibre in the wheat bran has on one hand beneficial health effects, but on the other hand it impedes the bioavailability of the wheat bran proteins. In a

study by Coda et al. (2014), wheat bran was micronized to different particle sizes and bioprocessed. In this study, bioprocessing was conducted with a starter culture, and with a starter culture and addition of enzymes. According to the study, the *in vitro* digestibility of proteins of both bioprocessed brans was higher compared to the native bran. The higher rate of *in vitro* digestibility may be caused by proteolysis by LAB and other micro-organisms, as suggested earlier by Clemente (2000) and Rizzello et al. (2013). Proteolysis by endogenous or exogenous proteases during fermentation may also have affected the higher digestibility of proteins. To determine the protein quality indexes, Coda et al. (2014) used the digestible protein fraction. The Essential Amino Acids (EAA) index indicates the ratio of EAA of the sample compared to the reference, whereas the biological value (BV) index estimates the nitrogen potentially retained by human body after consumption. Protein efficiency ratio (PER) is the protein efficiency ratio, which gives the ratio of gain in body mass in gram to protein intake in gram by a test subject. According to Coda et al. (2014), the EAA and BV indexes were the highest for both of the bioprocessed brans compared to the native brans. The addition of the enzymes improved the positive effect of the microbial fermentation for both indexes. The PER had the same trend. As stated in their study, the nutritional index (NI) is the only index, which combines qualitative and quantitative factors and is used as a global predictor of protein quality. Due to increased protein bioavailability, the NI of bioprocessed brans was higher compared to native bran. The difference was even higher when enzymes were added to the bioprocessed bran.

Wheat bran consists of dietary fiber and bioactive compounds such as alkylresorcinols, lignans, phenolic acids, phytosterols, tocopherols, tocotrienols, and folates (Liukkonen et al. 2003, Kamal-Eldin et al. 2009). According to Brouns et al. (2012), lignans occur as minor constituents in many plants. The physiological effects of lignans are mainly based on their antioxidant activity as well as on their potential estrogenic activity after transformation and absorption (Heinonen et al. 2001). Phenolic compounds of the wheat grain are located in the bran and protect the grain against oxidative damage (Anson et al. 2009). As stated in their study, FA is the most abundant phenolic compound in wheat bran, and these compounds make wheat bran a potential component for the production of nutritionally valuable cereal foods and new ingredients. According to Rizzello et al. (2008), bioprocessing increases the level of mineral bioavailability, bioactive peptides and solubility of dietary fibre. The use of bioprocessing techniques has given promising results in improving the bioaccessibility of nutritionally benefitting compounds in bran (Coda et al. 2014, Katina et al. 2007, Anson et al. 2009). The potential health effect of FA may partly be due to its antioxidant properties

(Srinivasan et al. 2007). According to Anson et al. (2009), bioprocessing of wheat bran by fermentation or by combination of hydrolytic enzymes and fermentation enhanced the release of phenolic acids and increased their free fraction in the wheat breads. In their study, the bioaccessibility of the phenolic acids was significantly increased by bioprocessing, and the bioaccessibility of FA was increased 5-fold by combination of fermentation and enzymatic treatment of wheat bran.

According to Coda et al. (2014), the amount of total phenolic compounds of bioprocessed bran increased after bioprocessing with enzymes. In this study wheat bran samples of different particle sizes were fermented for 24 h with selected LAB and added enzymes. Also the concentration of gamma-aminobutyric acid increased after bioprocessing as an effect of a lower pH. Bioprocessing also improved the amount of phytase. Partly the increasing was due to the smaller particle size of the bran, but as stated by Rizzello et al. (2010, 2012) phytase is provided by LAB during bioprocessing. As further stated in their study, phytase is important for degradation of phytate, which due to its strong mineral chelating properties reduces the bioavailability of the nutritional components.
According to Katina et al. (2012) bioprocessing of bran with enzymes and yeast increased the content of bioactive compounds in bread. Detrimental effects of bran on the mineral availability or on the technological performance of doughs could be overcome by using a combination of amylolytic and phytate-degrading enzymes, as stated in the study mentioned above.

Another health promoting aspect of whole grain consumption is according to (Chan et al. 2007, Mitrou et al. 2007 and Howard et al. 2008), a reduced risk of type-2 diabetes, cardiovascular disease, and some types of cancer, such as colonic cancer, pancreatic cancer, and small intestinal cancer. These health benefits are partly due to fibre and are linked to the formation of metabolic end-products by the microbiota of the intestinal tract, such as the short-chain fatty acids (SCFA). According to (Mateo Anson et al. 2009, Watzke, 1998), bioprocessing may result in structural modifications of the fibre affecting the fermentation properties in the colon.

2.3 Extraction and isolation of wheat bran proteins

Extraction and isolation of wheat bran proteins has been conducted previously, as reported by Fellers et al. (1966). In their article "solubilization and recovery of protein from wheat millfeeds" Fellers et al. describe a wet alkaline extraction of WB proteins to obtain a protein concentrate for human food. Some decades later Waszczynskyj et al. (1981) reported that a cellulase, hemicellulase, and pectinase treatment prior to alkaline extraction increased the protein extraction yield (that is, the percentage of total protein extracted) from 30% to 38.5%. Roberts and others (1985) further investigated the impact of the pH of extraction media, extraction time and temperature on the recovery of protein.

The main factors that affect the solubility of proteins are pH of the extraction media, isoelectric point (p*I*) of the protein and possible bonds of the proteins with other compounds, or intramolecular forces of the protein (Belitz et al. 2009). As stated by Belitz et al., the *pI* of a protein is a certain pH at which that protein carries no net electrical charge, and furthermore the solubility of proteins is lowest at their p*I*. At this point the protein in the solution precipitates out, and can be easily collected from the solution. At pH values differing from its p*I*, protein has a net charge. The presence of phytates in the wheat bran affects the solubility of proteins due to building of insoluble complexes between proteins and phytate at pH levels above and below their p*I*s (Guo et al. 2015). As described in their study, phytic acid (or phytate when in salt form) is the phosphate ester of inositol, and it is a storage form of phosphorus in many plant tissues, especially bran and seeds. Phytate has a strong binding affinity to the dietary minerals calcium, iron and zinc, inhibiting their absorption in the gastrointestinal tract (Guo et al. 2015).

According to de Rham and Jost (1979) and Grynspan and Cheryan (1989) below the p*I* of the protein, the anionic phosphate groups of phytate attach to the positively charged groups of protein. At pH values above their p*I*, both phytic acid and proteins have a net negative charge. At this pH however, it is possible for proteins to form complexes with phytic acid, due to the presence of multivalent cations. Grynspan et al. (1983) showed that at high pH (>10) the protein-cation-phytic acid compound dissociates and proteins stay in solution. Furthermore, alkaline conditions may assist the cleavage of hydrogen bonds and thus reducing aggregation and increasing the solubility of proteins (Hamada 1997).

Mechanical characteristics of the bran, as its size, affect the solubilization. According to Fellers et al. (1966), reducing the particle size of wheat bran (from 50% of the particles larger than 600 µm to about 90% smaller than 250 µm) increases the protein extraction yield with about 50% at alkaline pH. De Brier et al. (2015) also noted that a higher protein recovery can be obtained by using ground bran (<400 µm).

2.3.1 Alkaline extraction

Extraction of wheat bran proteins has mostly been conducted in alkaline media (De Brier et al. 2015, Balandrán-Quintana et al. 2015, Wang et al. 1999). Amongst the first published articles about extraction of wheat bran proteins is the comprehensive study by Fellers et al. (1966). Here, the solubilization and recovery of protein from millfeeds were investigated. Extraction was conducted at pH from 6.5–12.5 at 23 °C for 1 h. The solvent to millfeed ratio applied was 10 : 1. Recovery of proteins was obtained by precipitation of proteins at pH 5.5 at 23 °C. The precipitation pH was selected according to precipitation tests at a pH range from 3–7, where most of the wheat bran proteins precipitated at pH 5.5. After precipitation, the liquid was heated to 100 °C for 10 min. Heat inducted coagulation resulted to an additional yield of protein. After centrifuging, the precipitates were freeze dried directly. Extraction of coarse bran at pH 12.5 at 23 °C for 1 h resulted in a solubilization of protein of 84%. It should be noted that the coarse bran used in this study consisted of 20.6% of protein and 12.8% fibre, whereas the fine bran consisted of 22.0% of protein and 10.5% of crude fibre. Under the same circumstances the solubilization for fine bran and ground fine bran was 90% and 100% respectively. Highest yields of protein in the supernatant for ground coarse bran was obtained at pH 11.5, and for fine ground bran at pH 10.5, the yields being 68.8% and 71.3 % respectively. These values are based on amounts of total protein in the brans. This indicates, that the solubility is at its highest at pH 12.5, but the yield of supernatant protein is highest between pH 10.5 and 11.5. The protein yield of the isolates was 69% for fine bran and 86% for coarse bran.

In the study by Roberts et al. (1985) it has been stated clearly how a higher pH during extraction results in a higher protein yield. At pH 12, protein of the wheat bran was extracted with an 83% recovery (of available protein). As comparison, the protein recovery was 72% at pH 6.5. The bran used in this study consisted of 14.8% protein. The amount of fibre is not reported. In this study, the extraction was performed at 60 °C. This was validated by an experiment where extractions at pH 6.5 were conducted on a temperature range from 10 °C

to 90 °C. The highest yield was obtained at 60 °C. It was also stated, that the use of such a high extraction temperature would probably not be feasible on an industrial scale use, since the differences between the yields 10 °C and 60 °C were around 10%. The effect of pH of the extraction media and of the washing media was also studied. The pH of the washing media did not have an effect at pH 7 and pH 12. But if washed at a lower pH 2, the yield was also lower. Interestingly, the extraction time used in this study was a quite long one, 16 h. It was concluded, that the use of tap water in combination with a long extraction time of 16 h would be a feasible method for industrial use. According to the study, high pH has also its adverse effects, as vitamins as thiamin, pyridoxine and riboflavin are unstable at high pH

DeBrier et al. (2015) reported an extraction rate of 37% of total protein of the wheat bran protein when extracted at pH 12 for 1 h at 20 °C. A higher protein yield of 55% was obtained, when extraction temperature was raised to 60 °C, and the extraction was performed three times in a row. As a result of a modified Osborne fractionation of wheat bran protein, a total protein yield of 60% was obtained. In this method, the different protein fractions of the bran were extracted by selected solvents. Albumin and globulin were extracted with a 0.05 M sodium phosphate buffer containing 0.4 M NaCl. Prolamin was extracted with 60% ethanol, and glutelin was extracted with different solvents of which 0.05 M NaOH proved to be the most effective. The bran used in this study consisted of 16.9% protein, and 56% of fibre, of dry matter. The high amount of fibre might explain partly the lower extraction rate compared with the previous results.

Arte et al. (2015) used as well an alkaline extraction media, when extracting water- and salt soluble proteins from WB with a 0.05 M Tris-HCl buffer at pH 8.8. Due to their similar characteristics, also rice bran proteins have been extracted at alkaline pH. Alkali is the most commonly used solvent to extract rice bran proteins (Cagampang et al. 1966). According to Hamada (1997), the effectivity of the alkali solution to solubilize proteins is due to the ability of NaOH to break hydrogen, disulphide and amide bonds in proteins. Rice bran protein behaves accordingly to wheat bran protein, since the protein yield of rice bran increases with increasing pH of the extraction media, according to Bera and Mukherjee (1989). In their study they obtained a maximum solubility of rice bran protein at pH 9.0–10.5. An increase of nitrogen solubility was observed below and above the isoelectric point of rice bran protein (pH 4.5). Gupta et al. (2008) performed alkali extractions at a lower pH to avoid the detrimental effects of highly alkaline conditions on proteins. Instead other parameters as temperature were used to obtain higher protein yields. Their results show that at pH 9.5, as the extraction temperature was increased from 30 to 75 °C, the rice bran protein yield

increased from 21 to 48%. This result is in accordance with studies conducted on wheat bran proteins.

2.3.2 Extraction in varying pH

In a recent study DeBrier et al. (2015) extracted wheat bran proteins in a pH range of 2–12. The adjusted pH for the samples were 2, 4, 6, 8, 10 and 12. The pH adjustments were obtained by 0.1 M HCl for 2–6, and by 0.1 M NaOH for 8–12. The extraction rate was 12% for extractions below pH 7, which is clearly lower than the extraction rate of 37% for extraction at pH 12. Also Roberts et al. (1985) performed extractions in acidic conditions at pH 2 and pH 4.5. The pH was adjusted with 1 M HCl. The extraction temperature was 60 °C, and extraction time 16 h. In addition, the pH of the washing liquid was varied, being 2, 7 or 12. Extraction at pH 2 resulted in a protein recovery of 66%, when washed at neutral pH. Same result was true for extraction at pH 4.5. It is lower than the recovery of 83%, obtained by extraction at pH 12. According to Bera and Mukherjee (1989), at acidic pH, the increase of protein yields was only small because of the presence of phytates that form insoluble complexes with protein.

While extracting rice bran proteins in acidic media, Hourigan et al. (1997) reported that the use of Viscozyme L at 50 °C and pH 3.8 gave a protein yield of 57%. This was clearly higher than the alkali extraction yield of 47% for the same bran (Fabian et al. 2011). This increase in yield might as well be attributed to the use of enzymes. To produce a rice bran protein isolate, the defatted rice bran is mixed with water, adjusted to pH 5.0 and treated with phytase and xylanase to degrade phytic acid and break down the cell walls, respectively (Wang et al. 1999). The digested bran mixture is then adjusted to pH 10.0 to inactivate the enzymes and extract the proteins. After centrifugation the supernatant was adjusted to pH 4.0, precipitate was neutralized to pH 7.0 and freeze-dried as the rice bran protein isolate (Wang et al. 1999).

2.3.3 Enzymatic extraction

Some attempts to extract wheat bran proteins by enzymatic extraction have been made. In a patent “Method for the extraction of aleurone from bran” Bohm et al. (2011) mixed wheat bran with a particle size of 400–800 μm with water and stirred at a temperature of 45–55 °C.

An enzyme solution including xylanase, betaglucanase, cellulose and arabinose was added. According to the study, due to enzymatic degradation the aleurone cells can be first separated from the seed coat and then be dissolved. The dissolved aleurone cells and the cell contents can be separated from the remaining components by sieving. The wet sieved material therefore contained the contents of the aleurone cell wall fragments and of the cells. According to the patent, this material can be concentrated into a powder by spray drying and/or freeze drying. Of the extracted aleurone fraction 16% was protein. An analysis of the quality of the extracted protein was not presented in the patent.

A similar method has been applied for the extraction of rice bran proteins. Since no strongly alkaline extraction media is needed, no loss of nutritional characteristics was caused. According to Hourigan et al. (1997) the utilization of enzymes can make extraction of rice bran proteins easier in several ways. Carbohydrase can increase protein yield due to its ability to attack the cell wall components, and therefore liberate more protein from the polysaccharide matrix of bran (Hourigan et al. 1997, Wang et al. 1999). Since most of the carbohydrates in rice bran are cellulose and hemicellulose, cellulase and hemicellulase were used in degrading the bran matrix in the study by Hourigan et al. (1997). Enzymes such as phytase and α-amylase can aid in the extraction by breaking down bonds between proteins and starch and proteins and phytate, which otherwise would impede extraction (Wang et al. 1999). Phytase attacks the bond between protein and phytic acid. According to Wang et al. (1999), a yield of 57% of rice bran protein was obtained while using phytase in the extraction. A higher yield of 74.6% was obtained when both phytase and xylanase were used. Viscozyme L is a mixture of carbohydrases such as xylanase, cellulose, hemicellulase, and arabanase, which according to Guan and Yao (2008) can effectively break down linkages within the polysaccharide matrix and hence liberate more intercellular protein. Proteases to hydrolyse proteins to more soluble peptides. This ability has been used to obtain higher yields in protein extraction, and according to Hamada (1999), recovery of rice bran proteins have risen from 60 to 93% due to use of proteases.

2.4 Experimental design and modelling

Experimental design is a tool to conduct and plan experiments to obtain the maximum amount of information from the collected data by conducting the minimum of experiments (Leardi 2009). Prior to conducting an experiment, and selecting the proper model for it, one must first define the goal of the experiment. If an optimization is aimed for, quadratic terms need to be in the model (Lundstedt et al. 1998). With quadratic terms in the model, it is possible to determine non-linear relationships between the experimental variables and responses.

Response surface methodology (RSM) is an experimental methodology that analyses the relationship between the response and the independent variables (Bas et al. 2007). RSM is founded on a multitude of mathematical and statistical techniques. The name of this methodology is due to the possibility to graphically visualize the mathematical model it creates.
According to Bas et al. (2007) an optimization experiment where RSM is applied can be separated into three steps. First, the independent variables and their levels need to be determined. It is important to select the parameters that have major effects on the process. Second, the experimental design is selected, and the model equation predicted and verified. To be sure about the significance of the results, one must consider the fitness of the used model. The fitness of the model can be evaluated by parameters as the goodness of fit, the goodness of variation, model validity and reproducibility.
The goodness of fit (or explained variation) R2 shows the model fit and is the fraction of the total variation of the response that is explained in the model.
The or goodness of prediction (or predicted variation) Q2, shows an estimate of the precision of the future prediction, and is the fraction of the total variation of the response that can be predicted in the model. Values of R2 and Q2 are between 0 and 1. Acceptable values are dependent on the nature of the data that are being examined. According to Lundstedt et al. (1998) as a guideline, the following can be considered. For data of chemical nature, R2 is acceptable at ≥ 0.8, and Q2 acceptable at ≥ 0.5 and excellent at 0.8. Again for data of biological value, acceptable values for R2 are > 0.7 and for Q2 > 0.4. The difference between R2 and Q2 should not be more than 20%.
Model validity is a test of diverse model problems and is expressed as a value between 0 and 1. If a value less than 0.25 is obtained, an incorrect model might have been used. If that is

not the case, other possible problems might be the presence of outliers or a transformation problem.

Reproducibility is the variation of the replicates compared to overall variability. It should be greater than 0.5. According to Lundstedt et al. (1998) it is reasonable to refine the model prior to moving on with interpreting a satisfactory model. It is possible that both values of explained and predicted variation will increase in the process. In the refining process, the factors that are found to be insignificant in the coefficient plot are excluded. Factors having small piles within the boundaries of the confidence interval have no significance, while factors with bigger piles are more influential. It is important to notice, that deletion of the insignificant factors should always be made one at a time. The deletion of one factor may influence the confidence intervals of the remaining factors.

Last, a graphical presentation of the model is created, and the optimal operating conditions are determined. The graphical presentation of the model is created by the computer program, and can be seen as a three dimensional surface which is called the response surface plot. Also a two dimensional plot is created. It is called the contour plot. By interpreting these graphical presentations the relationship between the response and the independent variables, and the maximum and minimum points of a response are obtained.

RSM is a widely used methodology that has gained great popularity in chemistry-related sciences, food technology included (Bezerra et al. 2008, Granato et al. 2014). In food technology, RSM has previously been used in a variety of experiments from investigating optimized processes for improving bread quality to extraction of banana juice (Katina et al. 2005 and 2006, Lee et al. 2006, Flander et al. 2010).

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3 EXPERIMENTAL RESEARCH

3.1 Materials and methods

3.1.1 Wheat bran

Wheat bran used in this study was obtained by Lantmännen (Lantmännen Cerealia AB, Malmö, Sweden). The bran was received in three batches, the first batch arriving March 2016, the second batch arriving in April 2016, and the third batch arriving June 2016. The bran type used was Kruskakli, and had the following chemical composition according to Lantmännen: dietary fibre 54%, protein 14%, carbohydrates 11%, (of which 2.1% sugars), fat 6%, (of which 1.1% saturated fat). During the pre-experiments both coarse and milled bran was used. Particle size of the coarse and fine bran were analysed with the Mastersizer 3000 (Malvern Panalytical, UK). Particle sizes are shown in Figure 3. For milled bran, 90% of particles were smaller than 378 µm, and for coarse bran 90% of particles were smaller than 2330 µm.

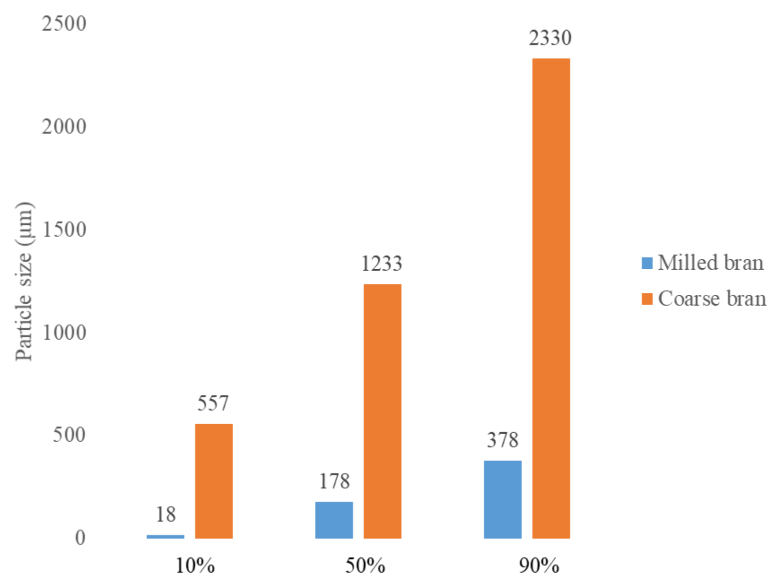

Figure 3. Particle size distribution of milled and coarse wheat bran. The percentages below the bars indicate the parts of bran, which have a particle size corresponding to the value presented, or below it.

3.2.2 Enzymes and starters

The enzymes used for bioprocessing of bran were Viscoferm (Novozymes, Bagsvaerd, Denmark) and Depol 761P (Biocatalysts Ltd., Wales, UK). A commercial starter culture including lactic acid bacteria *Lb.brevis* and *Lb.plantarum* and yeast *S. cerevisiae* (Florapan, LA4K, Lallemand, Montreal, Canada) was used for bioprocessing. The starter culture was added in a 1:1000 (w/w) ratio to bran. The dosage used for the enzymes Viscoferm and Depol 761P was during optimization 5, 50 and 500 nkat/g of bran, and afterwards 500 nkat/g. The main activities and the highest side activities of the selected enzymes are presented in Table 1.

Table 1. Main activities (m) and side activities of the selected enzymes. ND = not detected.

	Depol 761P (nkat/ml)	Viscoferm (nkat/ml)
xylanase	35828 (m)	33284
β-glucanase	1625	44317 (m)
cellulase	ND	13036
endoglucanase	ND	149
acidic protease (pH 5.0)	ND	2.5

3.2.3 Bioprocessing of bran

Two kinds of bioprocessed samples were prepared: samples with a starter culture, and samples with a starter culture and enzymes. The samples were prepared according to the experimental design (Table 2.). Wheat bran and milli-Q-water were mixed in beakers in a ratio of 30:70 (w/v), respectively. The enzymes and the starter were added to the beaker, and the slurry was mixed thoroughly with a spatula. The starter was added at a ratio of 1:1000 to wheat bran. The enzymes were added according to their main enzyme activity at doses of 5 nkt/g, 50 nkat/g and 500 nkat/g. The beaker was sealed with plastic film and aluminum foil. The beakers were placed in to incubators to the selected temperatures (20 °C, 27.5 °C or 35 °C) and incubated for 8 h, 16 h or 24 h. The incubations were performed in duplicate. After fermentation, the pHs of the samples were measured, and the samples (Figure 4.) were centrifuged at a speed of 10000 g for 20 min at 20 °C (Sorvall RC5C, Kendro Laboratory Products, North Carolina, United States) and the supernatant and precipitate was collected to obtain a protein-rich supernatant and a fibre-rich residue. For comparison, a control

sample was prepared by mixing bran and water (30:70 w/v) without incubation (time 0h) and addition of starters or enzymes. After mixing, the sample was centrifuged and the supernatant collected. The non-bioprocessed sample was prepared as above, but without added starters or enzymes.

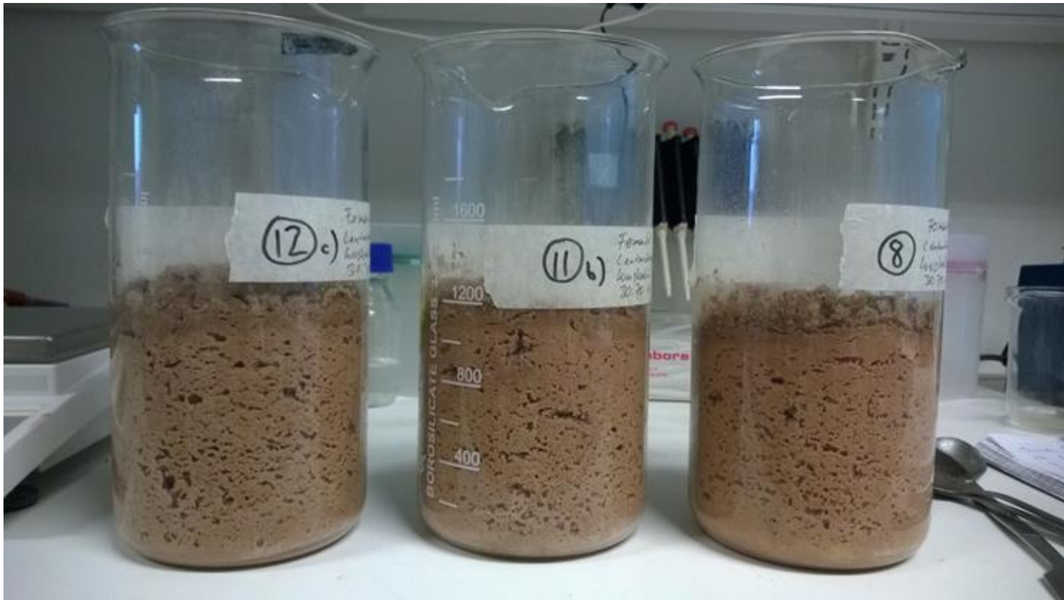

Figure 4. Bioprocessed wheat bran after fermentation.

3.2.4 Determination of soluble protein

Soluble protein content was analyzed by using the Kjeldahl method (AACC) 46-11A. For the analysis 1 ml of supernatant was weighed to the Kjelltec tube, and one Kjell tab and approximately 10 ml of 1 M HCl was added. The samples were heated at 400 ˚C for 1 h, and then let cool to room temperature. The tubes were closed with parafilm, and let at room temperature overnight. Next day, the samples were analyzed with the Kjeltec analysator (Foss Kjeltec 8100, Hilleroed, Denmark) to obtain the amount of nitrogen. The factor of 6.31 was used to convert the amount of nitrogen to crude protein (FAO 2003). The amount of soluble protein was calculated as the ratio of crude protein in the sample to the amount of maximum protein content in the wheat bran. In the later phases of the work, the protein content of the freeze dried supernatants and freeze dried residues was measured as well.

3.2.5 Determination of free amino nitrogen and peptide content

Free amino nitrogen was analyzed according to the method Free amino nitrogen of malt by spectrophotometry (Analytica-EBC, Verlag Hans Carl Getränke-Fachverlag, Nürnberg, 1998). The peptide content was determined by the o-phtaldialdehyde (OPA) method as described by Church et al. (1982).

3.2.6 Determination of pH and total titratable acids

For pH determination, a pH meter (Knick, Portamess 752 Calimatic, Germany) was used. Total titratable acids were measured using the Mettler Toledo EasyPlus titration system. In this method, 10 g of bioprocessed wheat bran slurry and 90 ml milli-Q-water was added to a 150 ml beaker. The electrode of the titration system was rinsed and placed to the sample. The titration of the sample was conducted under stirring conditions with 0.1 M NaOH solution. Measurement of pH was conducted to follow the optimal pH value for protein solubilization and protein quality. TTA was measured for the control sample and for selected bioprocessed samples.

3.2.7 Determination of soluble pentosan and reducing sugars

To get more information about cell wall degradation, soluble pentosan content and reducing sugar content was analyzed. Soluble pentosan content was analyzed as described by Santala et al (2011). In the pretreatment phase the bioprocessed wheat bran slurry was freeze dried. Then 0.25 g of freeze dried sample and 8 ml of milli-Q-water (at 4 ˚C) were vortexed shortly and then stirred for 15 min at 4 ˚C. Afterwards, the samples were centrifuged (4000 rpm, 20 min). The supernatant was poured to a new test tube and boiled for 15 min. After boiling, the samples were cooled in a water bath. Prior to analysis the samples were centrifuged (14000 rpm, 10 min). The amount of soluble pentosan was determined by a colourimetric phloroglucinol method by Douglas (1981). Xylose was used as a standard.

Reducing sugar content was estimated with the dinitrosalicylic acid method, as done by Bernfeld (1955). In this method, the diluted sample and the dinitrosalicylic reagent were

mixed in a test tube, and kept in boiling water for 5 min. After cooling in ice bath, the absorbance was measured with a spectrophotometer at 540 nm.

3.2.8 Electrophoresis

In order to understand changes in the molecular weight of solubilized proteins, the supernatants were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The samples were mixed at a ratio of 1:1 with SDS sample buffer and boiled for 5 min, and 10 µL of sample was pipetted to NuPage Bis-tris 10% gels (Invitrogen, Waltham, MA). Novex Sharp Prestained (10−250 mol/g, LC5800, Invitrogen, Waltham, MA) was used as a protein standard. The gels were stained overnight by Coomassie blue and destained with water.

3.2.9 Experimental Design and modelling

Modelling experiments were conducted with milled Kruskakli. Three factors (fermentation time, fermentation temperature and enzyme dosage) were selected for modelling. Fermentation time varied from 8 h to 24 h, fermentation temperature varied from 20 ˚C to 35 ˚C, and enzyme dosage from 5 nkat/g to 500 nkat/g. The samples were prepared according to the experimental design (Table 2). Amount of starter culture, water and wheat bran were kept at the same level throughout optimization. Optimization was conducted with MODDE software program 10.1 (Umetrics, Sweden), using central composite design of experiments and fitting the model was done with PLS (projection to latent structures).

Table 2. Composition of the various runs of the central composite design for bioprocessing with starter and for bioprocessing with starter and enzymes.

Bioprocessing with starter			Bioprocessing with starter and enzymes			
Run	Time (Ti, h)	Temperature (Te, °C)	Run	Time (Ti, h)	Temperature (Te, °C)	Enzyme dosage (E, nkat/g)
1	8	20	1	8	20	5
2	24	20	2	24	20	5
3	8	35	3	8	35	5
4	24	35	4	24	35	5
5	8	27.5	5	8	20	500
6	24	27.5	6	24	20	500
7	16	20	7	8	35	500
8	16	35	8	24	35	500
9	16	27.5	9	8	27.5	50
10	16	27.5	10	24	27.5	50
11	16	27.5	11	16	20	50
12	16	27.5	12	16	35	50
			13	16	27.5	5
			14	16	27.5	500
			15	16	27.5	50
			16	16	27.5	50
			17	16	27.5	50
			18	16	27.5	50

3.3 Results

3.3.1 Solubilized protein

An experimental design was performed to study the effects of time, temperature and enzyme dosage on the solubilized protein content. The goodness of fit (R^2) and goodness of prediction (Q^2) values were fairly high for samples with starters and for samples with strains and enzymes, respectively (Table 3). For samples with starters, the R^2 value was 0.86, and for samples with strains and enzymes 0.9. The lack of fit values varied between 0.042–0.051, and were thus close to the recommended values (>0.05). The model validity values varied between 0.21–0.25. The model validity value for samples with starters, 0.21, was somewhat below the recommended value (>0.25). Given the high values of goodness of fit and goodness of prediction, model validity should be adequate. High reproducibility values of 0.97–0.99 stated that the variation of the replicates was very small compared to variation of the experiment samples. The regression equations of the models are presented in Table 2.

Table 3. The regression equations and Summary of Fit statistics for the solubilized protein.

The regression equations and Summary of Fit statistics	Starters	Starters + enzymes
Regression equation	$1.9 - 0.02Ti - 0.02Te + 0.001Ti^2 + 0.0004Te^2$	$1.63 - 0.02Ti + 0.0001Te + 0.02E + 0.0005Ti^2 + 0.0002Ti*Te$
R^2	0.86	0.9
Q^2	0.66	0.7
Lack of fit	0.042	0.051
Model validity	0.21	0.25
Reproducibility	0.97	0.99

Ti = time (hours), Te = temperature (C°), E = enzyme dosage (nkat/g), Ti^2 = quadratic effect of time, Te^2 = quadratic effect of temperature, Ti*Te = time-temperature interaction.

For samples with starters the maximum amount of solubilized protein (>50%) was achieved after the longest fermentation time (24 h) and at the warmest temperature (35 C˚), as shown in Figure 5. The lowest amount of soluble protein (<38%) was at the point with a

fermentation time of 14 h, and temperature of 22 ˚C. Both fermentation time and temperature had significant effects on solubilized protein content, since the amount of soluble protein increased with longer fermentation time and higher temperature. The model coefficient plots for samples with starters are presented in Appendix 3.

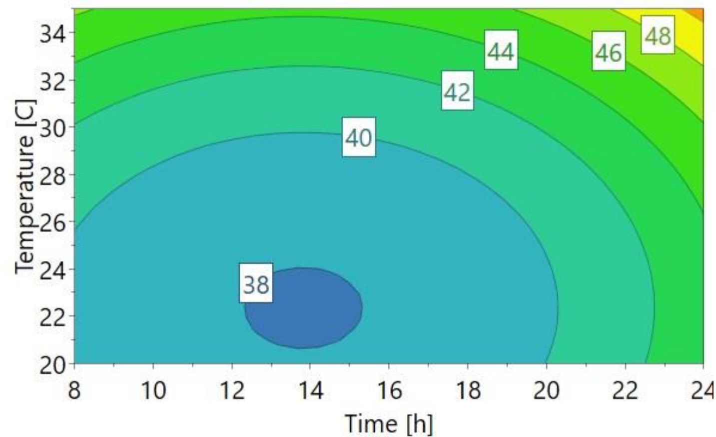

Figure 5. Impact of temperature (20-35 ˚C) and time (8-24h) on solubilized protein content in samples bioprocessed with starters.

For samples with strains and enzymes the maximum amount of solubilized protein (>50%) was also achieved after the longest fermentation time (24 h) and at the warmest temperature (35 ˚C), as presented in Figure 6. This was in accordance for all samples, irrespective of the enzyme dosage. The highest amount of solubilized protein was achieved with the highest enzyme dosage. Fermentation time, temperature and enzyme dosage significantly influenced the solubilized protein content. The model coefficient plots for samples with starters are presented in Appendix 4.

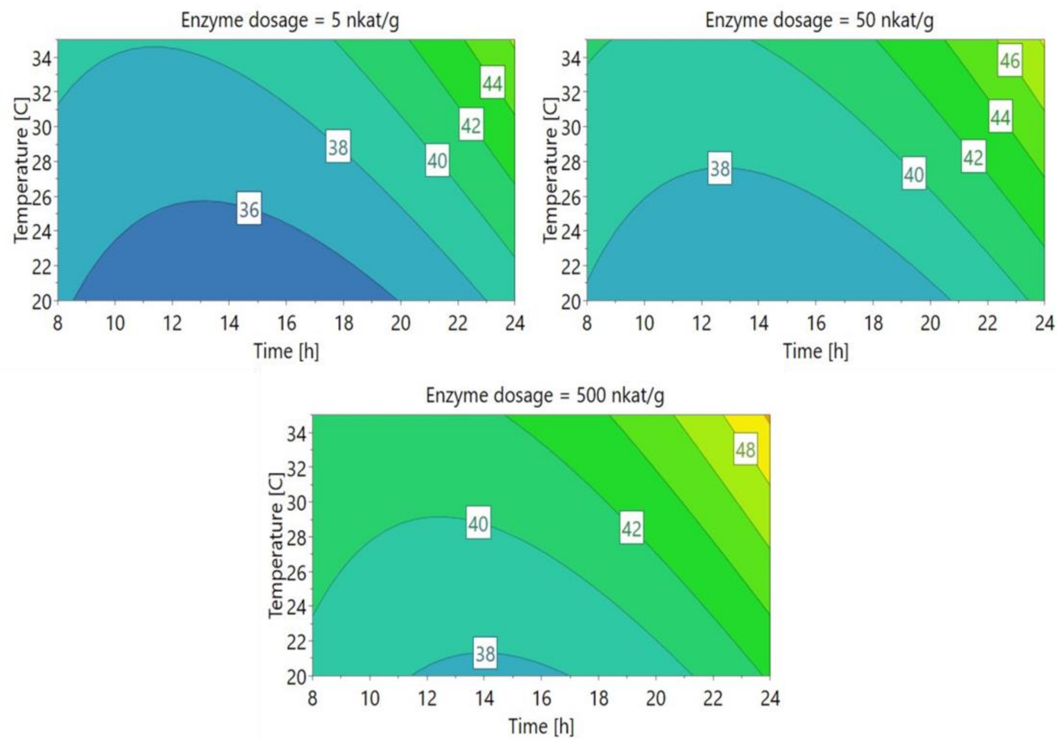

Figure 6. Impact of temperature (20-35 ˚C), time 8-24h and enzymes dosage (5-500 nkat/g bran) on solubilized protein content in samples bioprocessed with starters and enzymes.

3.3.2 pH and total titratable acidity

An experimental design was performed to study the effects of time, temperature and enzyme dosage on the pH. The models for pH seemed adequate, with goodness of fit (R^2) being 0.88 for both samples and goodness of prediction (Q^2) ranging from 0.64 to 0.77 for samples with starters, and for samples with starters and enzymes respectively (Table 4). Lack of fit and model validity, 0.033 and 0.15, respectively, were below recommendations for samples with starters. Lack of fit and model validity, 0.13 and 0.48, respectively, were above the recommended values for samples with starters and enzymes, and hence validate the model.

Table 4. The regression equations and Summary of Fit statistics for the pH.

The regression equations and Summary of Fit statistics	Starters	Starters + enzymes
Regression equation	$1.38 - 0.04Ti - 0.02Te + 0.001Ti^2 + 0.0003Te^2$	$1.14 - 0.04Ti - 0.005Te - 0.003E + 0.001Ti^2 + 0.0001Ti*Te$
R^2	0.88	0.88
Q^2	0.64	0.77
Lack of fit	0.033	0.13
Model validity	0.15	0.48
Reproducibility	0.97	0.97

Ti = time (hours), Te = temperature (C°), E = enzyme dosage (nkat/g), Ti^2 = quadratic effect of time, Te^2 = quadratic effect of temperature, Ti*Te = time-temperature interaction.

For samples with starters the highest pH level (6.5) was obtained with the shortest fermentation time and the lowest temperature, as indicated in Figure 7. As expected, the pH level was as lowest (<4) with higher temperature and longer fermentation time. As can be seen, the pH level started to drop soon after the temperature started to raise, and in process of time. Both time and temperature significantly influenced the pH level negatively, indicating the decrease of the pH level with longer time and warmer temperature (Appendix 3).

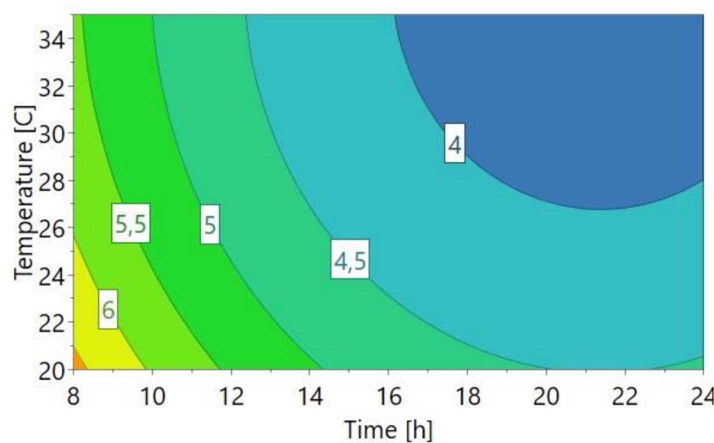

Figure 7. Impact of temperature (20-35 °C) and time (8-24h) on pH in samples bioprocessed with starters.

For samples with starters and enzymes the pH levels behaved according to the same trend as in bran bioprocessed with solely starters, as can be seen in Figure 8. The lowest temperature (20 °C) and shortest fermentation time (8 h) resulted in higher pH levels (>6.5), whereas high temperature and long fermentation time resulted in lower pH levels (<4.5). It should be

noted, that the lowest pH level was obtained with the samples with starters, where the lowest pH level was <4, compared to <4.5, which was the lowest pH level in the samples with starters and enzymes. No significant differences in the pH level occurred between the samples with different enzyme dosages. Both time and temperature significantly influenced the pH level negatively, indicating the decrease of the pH level with longer time and warmer temperature (Appendix 4).

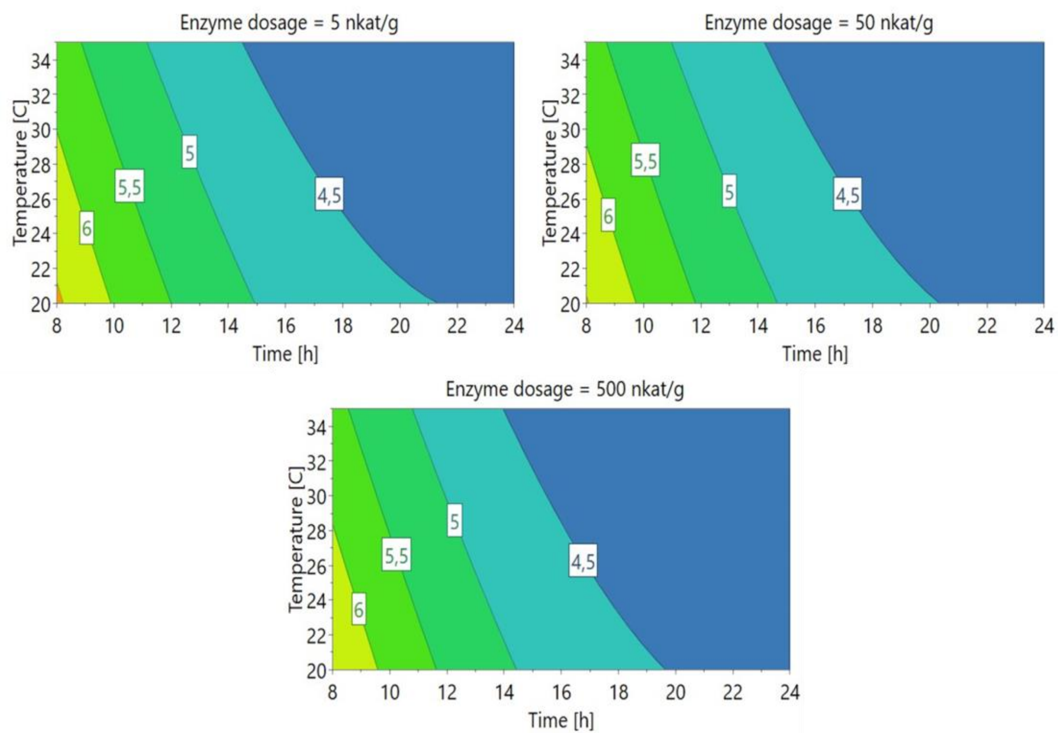

Figure 8. Impact of temperature (20-35 ˚C), time 8-24h and enzymes dosage (5-500 nkat/g bran) on pH in samples bioprocessed with starters and enzymes.

Total titratable acidity (TTA) of selected bioprocessed samples seemed to verify the results obtained by measuring the pH. The initial TTA level was 5.2 ml in non-bioprocessed bran as presented in Table 5. The TTA increased with longer fermentation time, and with the use of enzymes. TTA was highest (36.1 ml) in bran bioprocessed with starter and enzymes for 24 h at 35 ˚C, and lowest in bran bioprocessed with starter for 8 h at 35 ˚C.

Table 5. Total titratable acidity of selected bioprocessed samples and the non-bioprocessed sample.

Bioprocessing method	Time (h)	Temperature (˚C)	TTA (ml)
None	0		5.2
Starter	8	35	10.9
Starter	24	35	26.8
Starter + Enzymes	8	35	14.6
Starter + Enzymes	24	35	36.1

3.3.3 Free amino nitrogen (FAN) content

An experimental design was performed to study the effects of time, temperature and enzyme dosage on FAN content. The goodness of fit (R^2) and goodness of prediction (Q^2) were high for samples with starters and for samples with strains and enzymes (Table 6). The goodness of fit was 0.93 for samples with strains, and 0.96 for samples with starters and enzymes. The goodness of prediction was for samples with strains 0.71, and for samples with strains and enzymes 0.81, and therefore above the recommended value of 0.5 for a good model. The lack of fit value was 0.425 for samples with starters, and 0.036 for samples with strains and enzymes. The lack of fit and model validity values for starters and enzymes were somewhat below the recommended values of 0.05 and 0.25, respectively. The lack of fit and model validity values for strains were above the recommended values.

Table 6. The regression equations and Summary of Fit statistics for the free amino nitrogen

The regression equations and Summary of Fit statistics	Starters	Starters + enzymes
Regression equation	$1.88 + 0.01Ti + 0.03Te - 0.0001Ti^2 - 0.0003Te^2$	$2.14 - 0.004Ti + 0.006Te + 0.05E + 0.0003Ti^2 + 0.0003Ti*Te$
R^2	0.93	0.96
Q^2	0.71	0.81
Lack of fit	0.425	0.036
Model validity	0.79	0.17
Reproducibility	0.91	1

Ti = time (hours), Te = temperature (C°), E = enzyme dosage (nkat/g), Ti^2 = quadratic effect of time, Te^2 = quadratic effect of temperature, Ti*Te = time-temperature interaction.

The content of FAN for samples with starters was at its lowest (<250 mg/l) at 20 ˚C and 8 h fermentation time (Figure 9). With warmer temperature and longer fermentation time, the FAN content gradually increased up to its highest value (>450 mg/l) at 35 ˚C and 24 h fermentation time. Fermentation time and temperature significantly influenced the FAN content (Appendix 3).

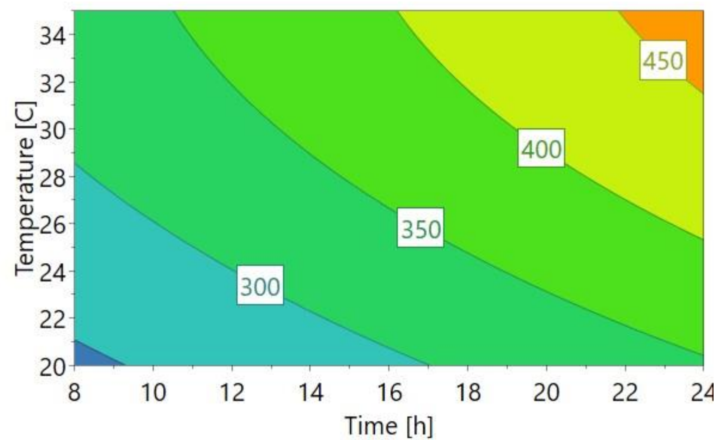

Figure 9. Impact of temperature (20-35 ˚C) and time (8-24h) on the FAN content in samples bioprocessed with starters.

The FAN content for samples with starters and enzymes followed the same trend as with samples for starters, as can be seen in Figure 10. The lowest FAN contents were obtained at the points with the lowest temperatures (20 ˚C) and shortest fermentation time (8 h). The overall trend was the same for all samples, regardless of the enzyme dosage. The FAN content was lowest in samples with the lowest enzyme dosage (5 nkt/g). This sample had also quite similar FAN contents compared to the samples with starters. The highest FAN content (>600 mg/l) was obtained in the sample with the highest enzyme dosage (500 nkat/g), at highest temperature (35 ˚C) and longest fermentation time (24 h). The samples with the enzyme dosage of 50 nkt/g placed between the samples with lower and higher enzyme dosages. Fermentation time, temperature and enzyme dosage had significant effects on the solubilized protein content (Appendix 4).

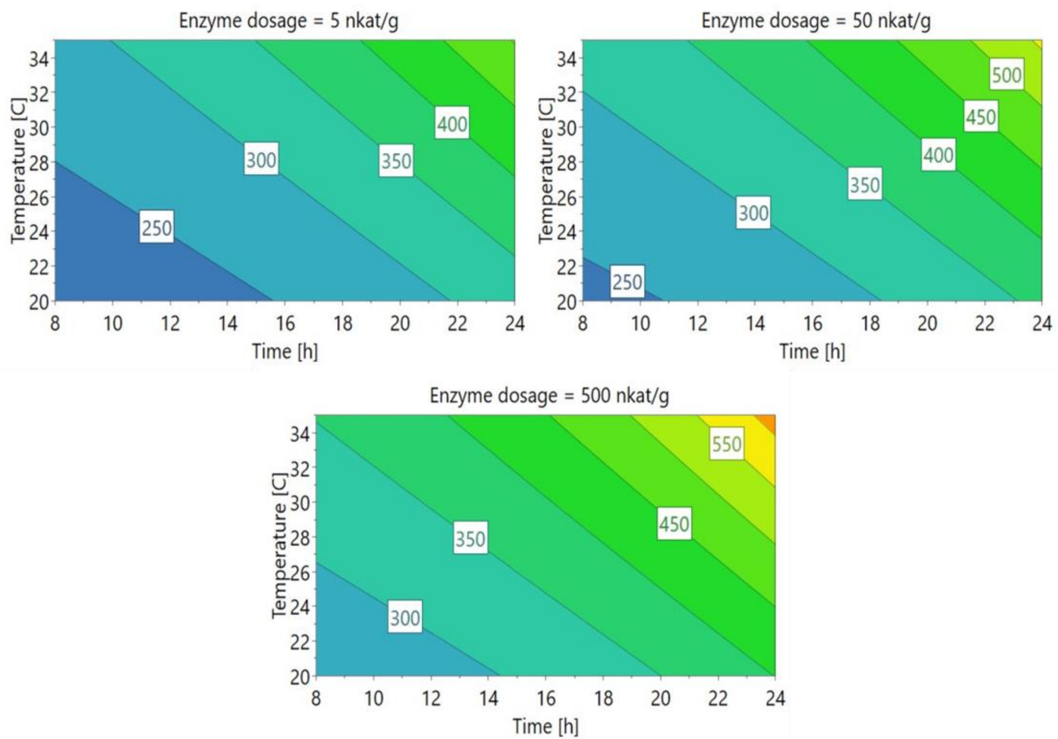

Figure 10. Impact of temperature (20-35 ˚C), time 8-24h and enzymes dosage (5-500 nkat/g bran) on FAN content in samples bioprocessed with starters and enzymes.

3.3.4 Peptide content

An experimental design was performed to study the effects of time, temperature and enzyme dosage on the peptide content. The goodness of fit (R^2) and goodness of prediction (Q^2) were fairly high, and exceeded the recommended values of 0.5, as can be seen in Table 7. The goodness of fit was 0.92 for samples with starters, and 0.95 for samples with starters and enzymes. The goodness of prediction was 0.77 for samples with starters and 0.8 for samples with starters and enzymes. The model validity and lack of fit values were very low (0.001 and -0.2, respectively).

Table 7. The regression equations and Summary of Fit statistics for the peptide content

The regression equations and Summary of Fit statistics	Starters	Starters + enzymes
Regression equation	1.17 - 0.01Ti - 0.01Te + 0.001Ti² + 0.0004Te²	0.99 - 0.02Ti + 0.004Te + 0.05E + 0.0006Ti² + 0.0003Ti*Te
R^2	0.92	0.95
Q^2	0.77	0.8
Lack of fit	0.001	0.001
Model validity	-0.2	-0.2
Reproducibility	1	1

Ti = time (hours), Te = temperature (C°), E = enzyme dosage (nkat/g), Ti² = quadratic effect of time, Te² = quadratic effect of temperature, Ti*Te = time-temperature interaction.

The peptide content for samples with starters was lowest (<12 mg/ml) at the point with lowest temperature (20 ˚C) and shortest fermentation time (8 h), as presented in Figure 11. With longer fermentation time and higher temperatures the peptide content increased, and reached its maximum (>20 mg/ml) at the point with the highest temperature (35 ˚C) and longest fermentation time (24 h). The peptide content was significantly influenced by fermentation time and temperature (Appendix 3).

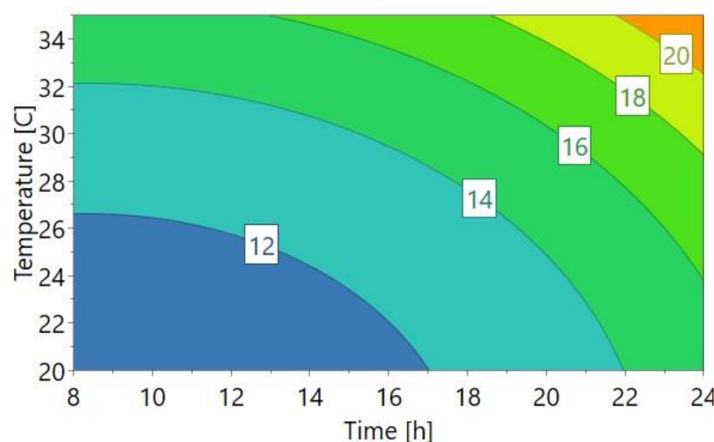

Figure 11. Impact of temperature (20-35 ˚C) and time (8-24h) on the peptide content in samples bioprocessed with starters.

The peptide content for the samples with starters and enzymes behaved accordingly to the samples with starters (Figures 11 and 12). The lowest peptide content (<12 mg/ml) was

obtained in the sample with the lowest enzyme dosage (5 nkat/g). This sample had quite similar peptide contents as the sample with starters. The highest peptide content (>26 mg/ml) was obtained in the sample with the highest enzyme dosage (500 nkat/g) at the point with the highest temperature (35 ˚C) and longest fermentation time (24 h). The samples with the enzyme dosage of 50 nkt/g placed as expected between the samples with lower and higher enzyme dosages. Fermentation time, temperature and enzyme dosage significantly influenced the peptide content (Appendix 4).

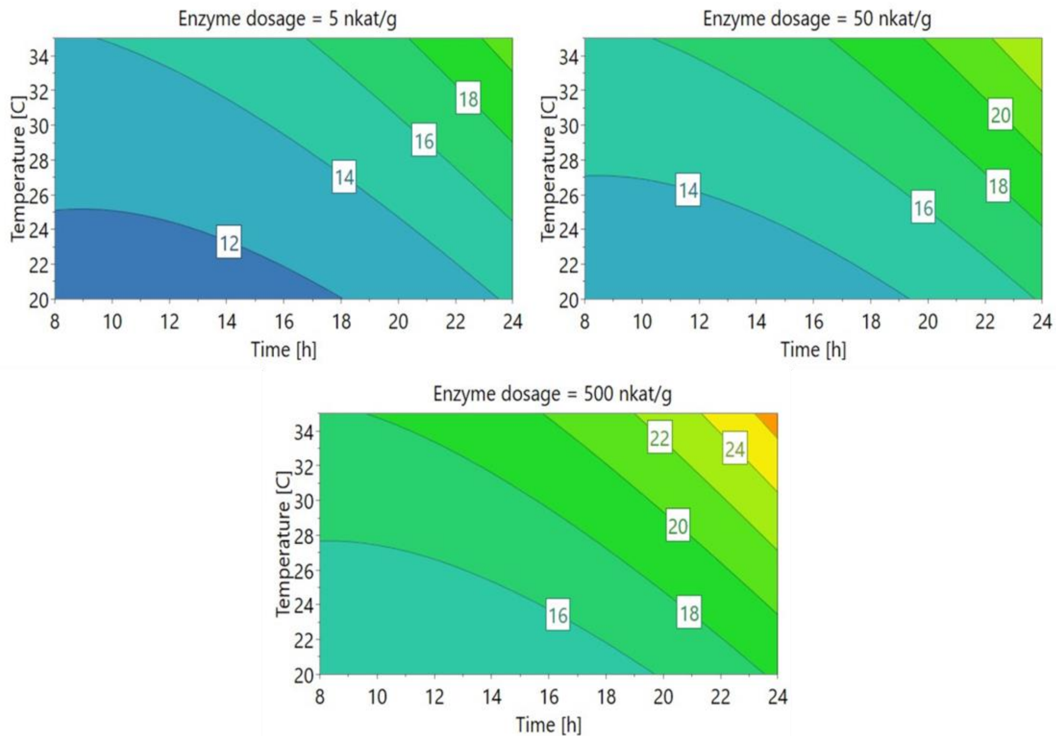

Figure 12. Impact of temperature (20-35 ˚C), time 8-24h and enzymes dosage (5-500 nkat/g bran) on the peptide content in samples bioprocessed with starters and enzymes.

3.3.5 Soluble pentosan

An experimental design was performed to study the effects of time, temperature and enzyme dosage on the soluble pentosan content. The goodness of fit (R^2) and goodness of prediction (Q^2) were low for samples with starters (0.58 and 0.22, respectively), as can be seen in Table 8. Whereas R^2 and Q^2 were fairly high for samples with starters and enzymes (0.98 and 0.84, respectively). The low values of goodness of fit and goodness of prediction for the samples with strains can be explained with the even so low reproducibility value (0.47). This indicates that the replicates of the samples have a large variability. On the other hand, the

model validity and lack of fit values were high for the samples with strains, being 0.41 and 0.78, respectively. These values indicate a valid model, with no evidence that the model would not fit the data. The lack of fit and model validity values were high for the samples with starters and enzymes (0.31 and 0.71, respectively). In combination with the high reproducibility value, this means that the model represents the data very well.

Table 8. The regression equations and Summary of Fit statistics for the soluble pentosan content.

The regression equations and Summary of Fit statistics	Starters	Starters + enzymes
Regression equation	-0.53 + 0.01Ti + 0.02Te - 0.0003Ti² - 0.0003Te²	0.25 - 0.03Ti - 0.002Te + 0.3E + 0.001 Ti² + 0.0002 Ti*Te
R^2	0.58	0.98
Q^2	0.22	0.84
Lack of fit	0.41	0.31
Model validity	0.78	0.71
Reproducibility	0.47	0.99

Ti = time (hours), Te = temperature (C°), E = enzyme dosage (nkat/g), Ti² = quadratic effect of time, Te² = quadratic effect of temperature, Ti*Te = time-temperature interaction.

The soluble pentosan content was lowest (<0.54%) at the point with the highest temperature (35 ˚C) and shortest fermentation time (8 h), as shown in Figure 13. A further point of low soluble pentosan content (0.56%) was found at the lowest temperature (20 ˚C) and shortest fermentation time (8 h). With increasing temperature and longer fermentation time the highest soluble pentosan content (>0.66%) was located to an area that begins at the point with a fermentation time of 21 h and temperature of 26 ˚C. From there, with the fermentation time approaching 24 h, the soluble pentosan content stays at its highest level, over a temperature range from 23 ˚C to 29 ˚C. Fermentation time had a significant effect on soluble pentosan content (Appendix 3).

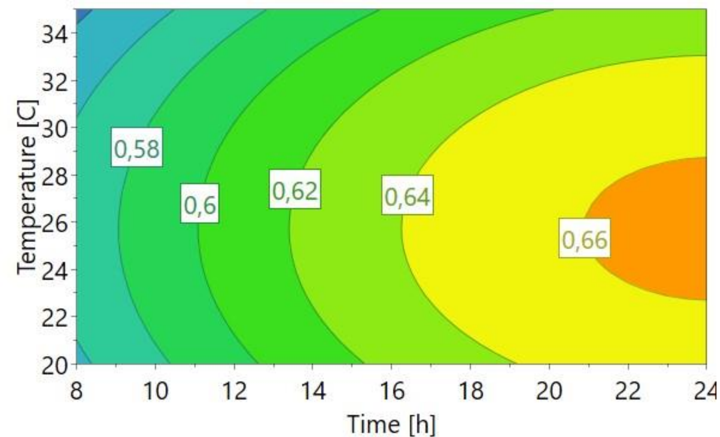

Figure 13. Impact of temperature (20-35 ˚C) and time (8-24h) on the soluble pentosan content in samples bioprocessed with starters.

The soluble pentosan content followed a different pattern for samples with starters and enzymes (Figure 14). The lowest soluble pentosan content (<1.9 %) was obtained with the lowest enzyme dosage of 5 nkat/g. The low content seemed to have a wide range in fermentation time between 10.5 h 19 h, and in temperature between 20 ˚C and 35 ˚C. The highest amount of soluble pentosan (>9.5 %) was found in the samples with the highest enzyme dosage (500 nkat/g). The point of the highest soluble pentosan content was located at the point with the highest temperature (35 ˚C) and longest fermentation time (24 h). The samples with the enzyme dosage of 50 nkt/g placed between the samples with lower and higher enzyme dosages. The soluble pentosan content was significantly influenced by the enzyme dosage (Appendix 4).

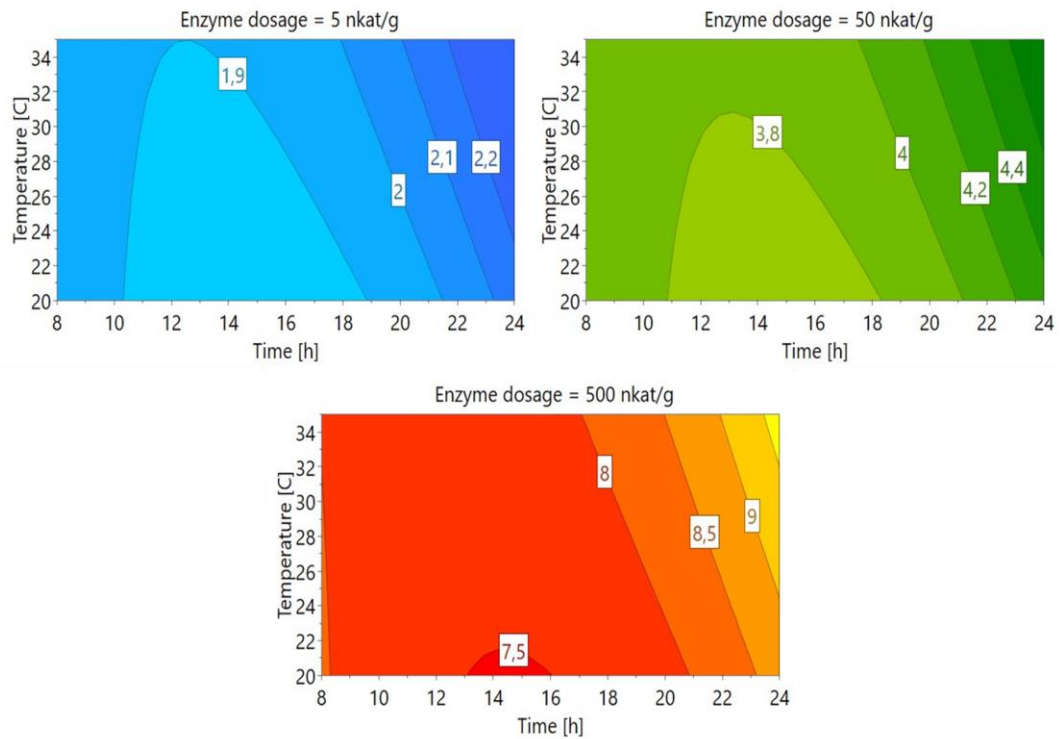

Figure 14. Impact of temperature (20-35 ˚C), time 8-24h and enzymes dosage (5-500 nkat/g bran) on the soluble pentosan content in samples bioprocessed with starters and enzymes.

3.3.6 Reducing sugars

An experimental design was performed to study the effects of time, temperature and enzyme dosage on the reducing sugars content. The goodness of fit (R^2) and goodness of prediction (Q^2) were above the recommended values for both samples, as presented in Table 9. For samples with starters R^2 was 0.84 and for Q^2 0.51. The lack of fit and model validity values were low (0.001 and -0.2, respectively), and clearly below the recommended values. The reproducibility value for the samples with starters was very high, and indicated the very small variability of the replicates. The samples with starters and enzymes had fairly higher R^2 and Q^2 values, 0.87 and 0.69 respectively. The lack of fit value (0.032) was somewhat below the recommended value of 0.05. The model validity value (0.13) was also below the recommended value of 0.25. The reproducibility value was then again very high (0.99).

Table 9. The regression equations and Summary of Fit statistics for the reducing sugars content.

The regression equations and Summary of Fit statistics	Starters	Starters + enzymes
Regression equation	124.98 - 3.63Ti - 5.08Te + 0.08Ti² + 0.08Te²	27.72 - 2.48Ti + 0.51Te + 15.58E+ 0.081 Ti² - 0.044 Ti*Te
R^2	0.84	0.87
Q^2	0.51	0.69
Lack of fit	0.001	0.032
Model validity	-0.2	0.13
Reproducibility	1	0.99

Ti = time (hours), Te = temperature (C°), E = enzyme dosage (nkat/g), Ti² = quadratic effect of time, Te² = quadratic effect of temperature, Ti*Te = time-temperature interaction.

The reducing sugars content was lowest (<5 g/l) at the point with the longest fermentation time (24 h) and highest temperature (35 ˚C), as presented in Figure 15. The highest reducing sugars content (>30 g/l) was found at the point with the shortest fermentation time (8 h) and lowest temperature (20 ˚C). Both fermentation time and temperature significantly influenced the reducing sugars content negatively (Appendix 3).

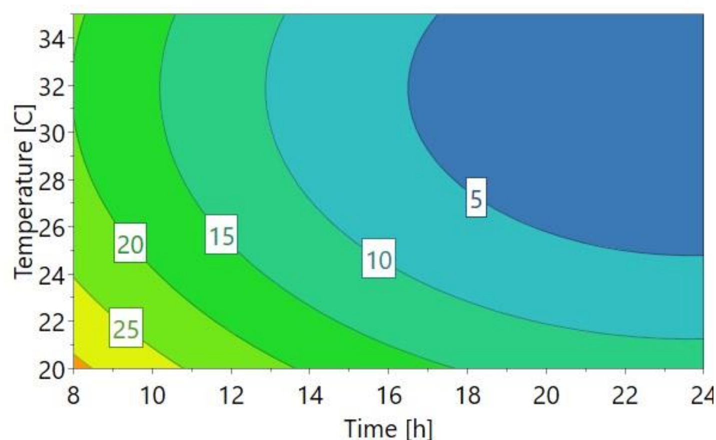

Figure 15. Impact of temperature (20-35 ˚C) and time (8-24h) on the reducing sugars content in samples bioprocessed with starters.

The reducing sugars content was lowest (<10 g/l) for the samples with the smallest enzyme dosage (5 nkat/g) at the point with the longest fermentation time (24 h) and the highest temperature (35 ˚C), as can be seen in Figure 16. The highest reducing sugars content (>60 g/l) was obtained with the samples with highest enzyme dosage (500 nkat/g) at the point with the shortest fermentation time (8 h) and with the highest temperature (35 ˚C). The

samples with the enzyme dosage of 50 nkt/g placed between the samples with lower and higher enzyme dosages. The enzyme dosage influenced significantly the reducing sugars content, whereas fermentation time influenced the reducing sugars content negatively (Appendix 4).

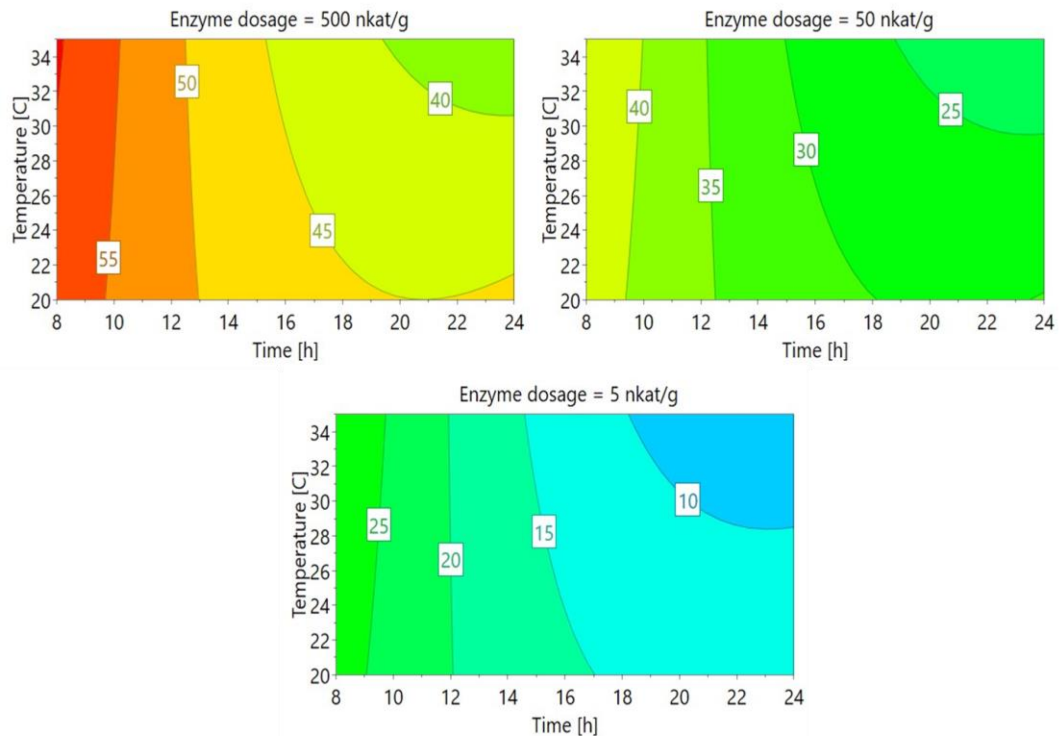

Figure 16. Impact of temperature (20-35 ˚C), time 8-24h and enzymes dosage (5-500 nkat/g bran) on the reducing sugars content in samples bioprocessed with starters and enzymes.

3.3.7 SDS-PAGE analysis of supernatants

SDS-PAGE analysis of non-bioprocessed bran supernatants showed an even distribution of proteins and peptides throughout the gel (Figure 17). As expected, no degradation of proteins was evident.

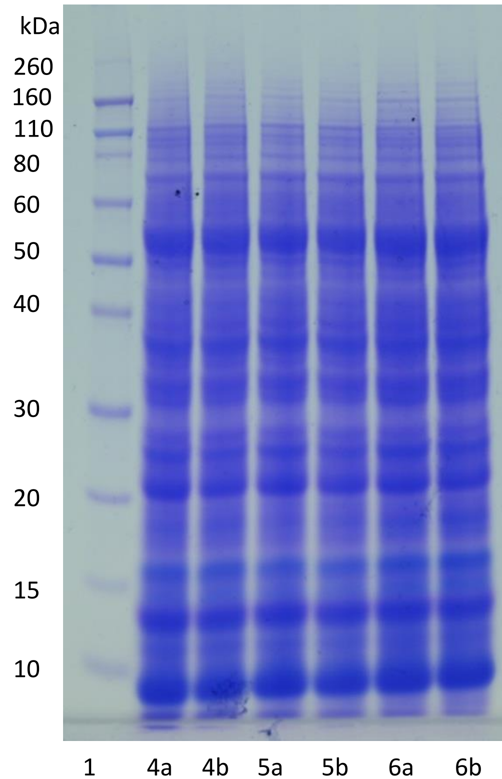

Figure 17. Quality of proteins in supernatants from non-bioprocessed bran. Lane 1 is the MW standard in kDa. 4: Non-bioprocessed bran (0 h) batch 1. 5: Non-bioprocessed bran (0 h) batch 2. 6: Non-bioprocessed bran (0 h) batch 3.

The quality of proteins of the brans bioprocessed with starter and with starter and enzymes for 8 h at 35 ºC were quite similar according to the SDS-PAGE analysis (Figure 18). The difference between these two samples and the non-bioprocessed samples were minor, with proteins in the range of 80–110 kDa being slightly degraded in the bioprocessed samples. Significant degradation of high molecular weight (HMW) proteins was evident in the bran bioprocessed for 24 h at 35 ºC. The degradation was stronger in the samples with starter and enzymes. The samples bioprocessed with starter for 24 h at 35 ºC had strong bands at 20, 25 and 30 kDa. The proteins larger than 40 kDa were completely degraded. The samples bioprocessed with starter and enzymes for 24 h at 35 ºC had a faint band at 20 kDa, but the larger proteins were completely degraded.

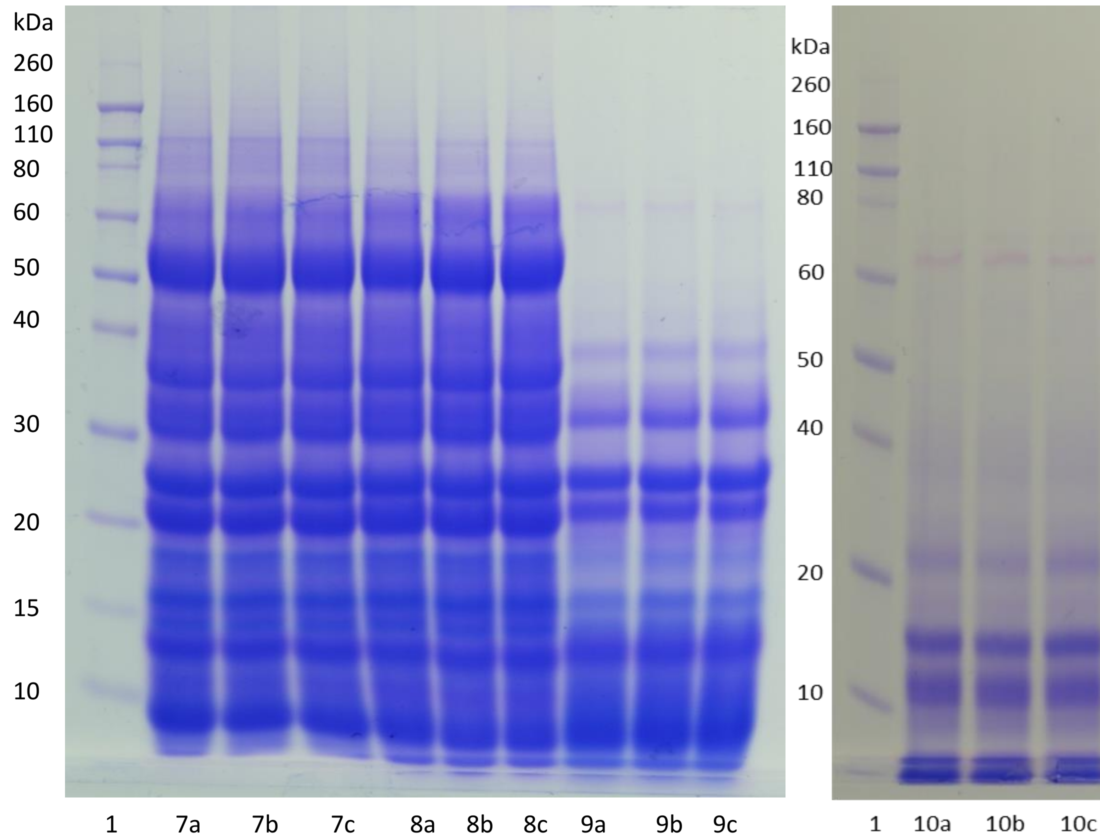

Figure 18. Quality of proteins in supernatants from bioprocessed bran. Lane 1 is the MW standard in kDa. 7: Bran bioprocessed with starter (8 h; 35 ºC). 8: Bran bioprocessed with starter and enzymes (8 h; 35 ºC). 9: Bran bioprocessed with starter (24 h; 35 ºC). 10: Bran bioprocessed with starter and enzymes (24 h; 35 ºC).

3.4 Discussion

3.4.1 Bioprocessing with starter culture

The highest protein solubilization (>50%) was achieved after the longest fermentation time (24 h) and highest temperature (35 ˚C), whereas the lowest amounts of solubilized protein (<38%) were found at the fermentation time of 14 h, and temperature of 22 ˚C according to the experimental desing. Similar results regarding protein solubilization have been reported by Arte et al. (2015 and 2016). In the study by Arte et al. (2015) wheat bran was bioprocessed at 30 ˚C for 24 h. It is important to note that the wheat bran used in the study was different from the bran used in this study. The wheat bran was coarse and hence the particle size of the bran might contribute to differing results, when compared with this study. The starter used by Arte et al. (2015) was also a different then the starter used in this study. According to Arte et al. (2015) the amount of solubilized protein for the sample bioprocessed with starter culture was 52.1%, which corresponds to the results obtained in this study. In the study by Arte et al. (2016) they bioprocessed wheat bran without added starter culture at 37 ˚C for 16 h, and the amount of solubilized organic nitrogen was 42.8%. The somewhat lower amount might be explained with a slower acidification process due to the lack of added LAB, which in turn leads to slower activation of endogenous proteolytic enzymes (Loponen et al. 2004, Gänzle et al. 2008). The wheat bran used in the study by Arte et al. (2016) was milled and had a mean particle size of 160 µm, which corresponds to the wheat bran used in this study.

To activate the enzymes of the bran, pH has to decrease to the optimal area of their activity. Acidification due to the growth of LAB decreases pH and activates endogenous enzymes of the wheat bran, as xylanases, arabinofuridases and aspartic proteases (Loponen et al. 2004, Carson et al. 2009, Katina et al. 2012). Endogenous proteolytic enzymes of the wheat bran degrade proteins to peptides (Thiele et al. 2004, Gänzle et al. 2008). In wheat bran, the endogenous proteolytic activity is mainly due to aspartic proteinases and serine carboxypeptidases, and the optimal pH level for these enzymes is 4–4.5 (Breddam et al. 1987, Gänzle et al. 2008). This is in accordance with the obtained pH results. The pH decreased with longer fermentation time and higher temperature. The higher solubilization of proteins occurred when the pH level was under 4.5. Similar results regarding the pH level have been reported by Arte et al. (2015 and 2016). In the 2015 study, the pH of the samples bioprocessed with strains was 4.3 after 24 h. The same pH level of 4.3 was measured for the

samples bioprocessed without added lactic acid bacteria in the 2016 study. The decrease in pH in both studies to the optimal level of endogenous carboxypeptidases justifies the rise in protein solubility at least partly due to the activity of the proteases. The solubilized protein content was measured using the Kjeldahl method. Since the Kjeldahl method measures the amount of organic nitrogen, it might overestimate the total amount of proteins, as there are other nitrogen containing compounds present in the wheat bran (Moore et al. 2010). The high solubilisation of proteins after the longer fermentation time (24 h) might also have been affected by production of microbial protein due to the growth of lactic acid bacteria.

The FAN content and peptide content were measured to study the degree of proteolysis. The content of FAN was significantly influenced by fermentation time and temperature. The lowest FAN content (<250 mg/l) was obtained at the shortest fermentation time (8 h) and lowest temperature (20 ˚C). Similarly the highest amount of FAN (>450 mg/l) was at the point with the longest fermentation time (24 h) and highest temperature (35 ˚C). The same trend applied for the peptide content, where the highest amount of peptides (>20 mg/ml) was obtained at the point with the longest fermentation time (24 h) and highest temperature (35 ˚C). The results regarding the peptide contents are in correspondence with earlier findings (Arte et al. 2015), where the peptide content of wheat bran bioprocessed with starter culture at 30 ˚C for 24 h was reported to be 13.2 mg/ml, compared to (>20 mg/ml) in this study. No studies with FAN analysis of solubilized wheat bran proteins were found. However, as previously pointed out by Gänzle et al. (2008), the degradation and depolymerization of proteins during fermentation is dependent on bacterial metabolic activity and cereal enzymes. Further, the activation of the proteolytic enzymes of the wheat bran depends on the appropriate pH level, and thus on time. Accordingly, the high amount of FAN after the longer fermentation time and at high temperature seem reasonable. The use of SDS-PAGE analysis delivered further knowledge about the degradation of proteins. The protein quality of bran bioprocessed with starter obtained by SDS-PAGE analysis was similar to the SDS-PAGE profiles of bioprocessed wheat bran conducted by Arte et al. (2015 and 2016). The differences between short time fermentation (4 h and 6 h) and long time fermentation (16 h) by Arte et al. (2016) highlight the same trend as seen between the 8 h and the 24 h samples of this study. With longer time, the endogenous proteolytic enzymes have more time to degrade proteins into smaller parts, which can be seen as a lack of bands in the area of larger MW proteins of the gels. Since one of the aims of this work was to liberate protein from the bran matrix without extensive degradation, it is of importance to highlight the points with lower degradation.

Soluble pentosan and reducing sugars were measured to follow the degradation of the aleurone cell walls. The content of WE pentosan represents hydrolysis products from AX, such as arabinose and xylose, and the content of reducing sugars represents hydrolysis products from β-glucan and cellulose. Since the pentosans are not a physical entity, it should be mentioned that the pentosans of importance in this study were the arabinoxylans and arabinogalactans. The highest soluble pentosan content (>0.66%) was at the fermentation time of 24 h and temperature of 26 ˚C. The lowest content of soluble pentosans (<0.54%) was found at the point with highest temperature (35 ˚C) and shortest fermentation time (8 h). These contents do not differ much, regarding the long range of time from 8 h to 24 h. Also, the values of goodness of fit and goodness of prediction (Table 7) for the model of soluble pentosan were considerably low, which indicate a poor model. This might be explained by a so-called uncontrolled factor, which in this case supposedly is due to the metabolism of LAB, where the bacteria use solubilized pentosans as carbon source during the growing stage of the microbes. The utilization of hydrolysis products from fibre compounds during LAB fermentation has been previously reported by Katina et al. (2005). As is known with the endogenous proteolytic enzymes, a decrease of pH initiated by LAB fermentation also activates endogenous degrading enzymes which hydrolyze fibrous compounds to sugars, which become available for fermentation by the LAB. Such enzymes are amylase, pentosanase, and β-glucanase. With longer fermentation time, there are more soluble pentosans available due to the hydrolyzing activity conducted by the endogenous carbohydrases (Katina et al. 2005). Accordingly with longer fermentation time the amount of LAB increases, as there are sufficient amounts of nutrients, partly in form of soluble pentosans. The soluble pentosan contents measured in this work align with earlier findings by Arte et al. (2015). There, wheat bran bioprocessed with starter at 30 ˚C for 24 h had a soluble pentosan content of 0.9%, compared to 0.66% in this study.

The highest amount of reducing sugars (>30 g/l) was obtained at the point with the shortest fermentation time (8 h) and with the lowest temperature (20 ˚C), whereas the lowest amount (<5 g/l) was found to be at the point with the longest fermentation time (24 h) and highest temperature (35 ˚C). The results obtained by Arte et al. (2015) were different, giving a reducing sugar content of 1.7 g/l for wheat bran bioprocessed at 30 ˚C for 24 h. This difference could be explained with the use of a different starter culture, since the LAB and yeasts consume reducing sugars during long fermentation times. Also the amount of used starter culture was different, and probably affected the consumption of reducing sugars by LAB and yeasts. The reducing sugars content was higher after short fermentation (8 h), than

after long fermentation (24 h), whereas the amount of soluble pentosan remained almost constant despite of the fermentation time. The same trend was reported by Arte et al. (2016), where soluble pentosan and reducing sugars contents were highest after 4 h and 6 h of bioprocessing, compared to the contents after 16 h of bioprocessing. This suggests that the soluble pentosans and reducing sugars were to a large part used as nutrients by bacteria and yeasts in their metabolism.

3.4.2 Bioprocessing with starter culture and enzymes

The highest protein solubilization (>50%) was achieved after the longest fermentation time (24 h) and highest temperature (35 ˚C). The long fermentation time enables the pH to decrease to an appropriate level for the endogenous and exogenous enzymes to activate and initiate their hydrolytic work. The enzyme dosage influenced the solubilization of proteins. As expected, the highest solubilization of proteins occurred with the highest enzyme dosage. This means that a larger amount of enzymes results to a more extensive degradation of the cell wall components. Accordingly, the protein solubilization was lower in the samples with lower enzyme dosages. These results are in accordance with the results obtained by Arte et al. (2016). In this study Arte et al. (2016) bioprocessed wheat bran for 16 h at 37 ˚C with addition of different enzymes with an enzyme dosage of 100 nkat/g. The amount of solubilized nitrogen obtained with the Kjeldahl method was 46.3% and 45.1% for bran treated with Depol 761P and bran treated with Viscoferm, respectively. The somewhat lower amounts of solubilized nitrogen might be due to a shorter fermentation time. Interestingly though, the amount of solubilized protein did not differ significantly from the amount of solubilized protein that was obtained by using only starter culture in bioprocessing. This seems to indicate that the major factor in protein solubilization is the activity of the endogenous enzymes of the bran. The same phenomenon was recognizable in the study by Arte et al. (2015), where wheat bran bioprocessed with starter culture had the same amount of solubilized protein as the bran bioprocessed with starter culture and enzymes. In the same study the highest amount of solubilized protein was obtained by the bioprocessing method where only endogenous enzymes were used. This definitely emphasizes the strong impact of the endogenous enzymes on the protein solubilization. When lower enzyme dosage levels of 5nkat/g and 50 nkat/g were used, the protein solubilization was lower when compared to the bioprocessing method using starter culture only. This indicates that the exogenous enzymes are effective only after a certain critical dosage level is exceeded.

The pH level decreased with longer fermentation time and higher temperature. This decrease in pH is probably due to the metabolism of the LAB during bioprocessing, since the end products of the fermentation are acetic acid and lactic acid.

The content of FAN increased with the fermentation time, temperature and enzyme dosage. Accordingly, the highest FAN content (>600 mg/l) was obtained at the point with the longest fermentation time, highest temperature and highest enzyme dosage. It seems, that the addition of carbohydrolytic enzymes impacted the degradation of proteins. It is suggested that the addition of carbohydrases accelerates the acidification through delivery of fermentable sugars for LAB metabolism, which in turn leads to a more intensive fermentation (Arte et al. 2015, Coda et al. 2014a and 2014b). The amount of FAN was significantly higher with the enzyme dosages of 50 nkat/g and 500 nkat/g, when compared to the bran bioprocessed with starter only. However the amounts of FAN were similar for the bran with enzyme dosage of 5 nkat/g and the bran bioprocessed with starter only. This indicates that the more extensive proteolysis through the addition of the enzymes was initiated first at a certain level of enzyme dosage, which in this case is somewhat below 50 nkat/g.

The peptide content followed the same pattern as the FAN content, with the highest peptide content (>26 mg/ml) obtained at the highest temperature, longest time and highest enzyme dosage. This is presumably due to the effect of fast acidification initiated by the carbohydrolytic work by the added enzymes. Similarly, the peptide content of the bran bioprocessed with starter only was at the same level compared to the one with an enzyme dosage of 5 nkat/g. Accordingly, the bran with an enzyme dosage of 50 nkat/g had only a slightly higher peptide content than the bran bioprocessed with starter only. This indicates the impact of the sufficient amount of enzyme dosage.

The differences in protein quality obtained by SDS-PAGE analysis between the shortest and longest fermentation time were significant. The intensive degradation of HMW proteins after 24 h was similar with the SDS-PAGE profile by Arte et al. (2015), where bands in the range of 18 - 32 kDA were obtained for the bran bioprocessed with starter and enzymes for 24 h. As expected, the degradation after 8 h of bioprocessing was weaker, and no differences were significant between the bioprocessing methods for the 8 h samples.

The soluble pentosan content was highest at the point with the longest fermentation time and highest temperature, as expected. Furthermore, the enzyme dosage influenced significantly the solubilization of pentosan, with the highest amount being solubilized with the highest

enzyme dosage. This seems reasonable, since the addition of the enzymes indeed increases the total amount of cell wall degrading enzymes. In addition to endogenous enzymes, now also exogenous enzymes participate in the hydrolysis of the fibrous cell wall components. The amounts of soluble pentosan were higher for all brans bioprocessed with starter and enzymes, compared to the ones bioprocessed with starter only. It is notable, that the values of goodness of fit and goodness of prediction for bran bioprocessed with starter and enzymes were high and indicated a strong model, as opposed to bran bioprocessed with starter only. Presumably this change is due to the significantly higher amount of soluble pentosans in the brans bioprocessed with starter and enzymes. This would mean, that even though the LAB start using soluble pentosans and reducing sugars as nutrients, the amount of the soluble pentosans and reducing sugars is so high, that it does not impact the summary of fit statistics of the model. The enzymes Viscoferm and Depol 761P were selected due to their main activities, which are β-glucanase and xylanase, respectively. These enzymes have been used separately and in combination previously to degrade the cell wall matrix of the wheat bran (Santala et al. 2011 and 2013, Arte et al. 2015 and 2016). Micrographs of bioprocessed wheat brans with Viscoferm and Depol 761P separately showed thinner aleurone cell walls and indicated hence moderate degradation of cell wall components (Arte et al. 2016), with the enzyme dosage of 100 nkat/g. The combined use of Viscoferm and Depol 761P and starter culture in bioprocessing of wheat bran for 24 h at 30 ˚C with an enzyme dosage of 100 nkat/g resulted in a soluble pentosan content of 1.9% (Arte et. al 2015). This is somewhat lower than the soluble pentosan contents obtained in this study for enzyme treated bran. For bran bioprocessed with enzymes and starter with the enzyme dosage being 5 nkat/g, the soluble pentosan amount was 2.3%. As already discussed above the difference might be due to the different starter culture used in the study by Arte et al. (2015), and hence the metabolism of the LAB and yeasts. The difference might to some extent also be due to the warmer bioprocessing temperature used in this study.

The reducing sugars content behaved similarly to the reducing sugars content of the bran bioprocessed with starter only. However, the highest content for brans bioprocessed with enzymes was at the point with shortest fermentation time and highest temperature, whereas it was for bran bioprocessed with starter only at the point with the shortest fermentation time and lowest temperature. As expected, the enzyme dosage affected the reducing sugars content, with the highest contents being obtained with the highest dosage. The obtained results differed clearly from the results obtained by Arte et al. (2015), where the amount of reducing sugars after bioprocessing for 24 h at 30 ˚C with the enzyme dosage of 100 nkat/g

was 0.027 g/l. In contrast, the amounts obtained in this study were 25 g/l and 40 g/l for enzyme dosages of 50 nkat/g and 500 nkat/g, respectively. As postulated above, the differences might be due to the use of a different starter culture and yeasts in the study by Arte et al. (2015). Furthermore, the reduction of the reducing sugars content with time is presumably due to the LAB metabolism, where reducing sugars are consumed during longer fermentation periods. It is noteworthy, that only the amount of reducing sugars decreased, whereas the amount of soluble pentosans increased over time. This phenomenon could be explained by the twofold higher initial amount of AX in the wheat bran compared to the amount of cellulose. Also, the preferred LAB metabolism pathways might play a role in which order nutrients are consumed by the LAB (Gobbetti 1998 and Gänzle et al. 2007).

In conclusion, the degradation of wheat bran fibres with bioprocessing was possible to achieve. While considering the amounts of solubilized protein, it is clear that the degradation of the prevalent fibre components in the aleurone cell wall was only partial. The main reasons for the partial degradation of wheat bran fibres probably were related to the utmost complex and strong structure of the aleurone cell wall (Bacic and Stone 1981, Rhodes et al. 2002, Saulnier et al. 2007). Furthermore, the presence of ferulic acid reinforces the structure due to building of cross links, and the presence of endogenous xylanase inhibitors in the wheat bran might hinder the proper functionality of the commercial enzymes used (Jerkovich et al. 2010 and Berrin and Juge 2008).

4 CONCLUSIONS

The aim of this thesis was to create an optimized bioprocessing scheme to solubilize the maximal amount of proteins, without losing their technological and nutritional quality. Optimized bioprocessing of wheat bran led to maximal protein solubilization of >50% for wheat bran both bioprocessed with starter culture and with starter culture and enzymes after fermentation time of 24 h. This indicates that the use of enzymes did not effectively break down the aleurone cell walls for protein liberation. This is supposedly due to the complex and strong structure of the aleurone cell walls, and the presence of compounds such ferulic acid and endogenous xylanase inhibitors. Furthermore the use of enzymes increased heavily protein degradation for fermentations longer than 8 h. Since the amount of solubilized protein was higher for wheat bran bioprocessed for 8 h with starter only (>46%) than for wheat bran bioprocessed for 8 h with starter and enzymes (>40%), the use of enzymes for a larger scale production does not seem feasible. Furthermore, the fermentation time should be considered when planning a feasible process. According to this study, it seems that the benefits of a longer fermentation time for higher protein solubilization are lost due to the detrimental effects of protein degradation during longer fermentation times.

For future experiments, several factors should be taken into account. Since wheat bran is not an uniform raw material, to successfully solubilize as much proteins as possible one should select a wheat bran with low fibre and high protein content. Furthermore, the particle size of wheat bran should be small, since it increases the solubilization of proteins. A more detailed selection of enzymes could be beneficial, especially if enzymes with resistance towards endogenous xylanase inhibitors can be found. Additionally, another method for protein quantification could be used, to get even more realistic results of the solubilized protein content.

ABBREVIATIONS

AX	Arabinoxylan
A/X	Arabinose to xylan ratio
BV	Biological value
EAA	Essential amino acid
FA	Ferulic acid
FAN	Free amino nitrogen
HMW	High molecular weight
LAB	Lactic acid bacteria
MW	Molecular weight
NI	Nutritional index
PER	Protein efficiency ratio
p*I*	Isoelectric point
RSM	Response surface methodology
SDS-PAGE	Sodium dodecyl
TTA	Total titratable acid
WE pentosan	Water-extractable pentosan

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APPENDICES

Appendix 1. Bioprocessing conditions and measured values for responses for samples with strains.

	Bioprocessing conditions		Response					
Sample	Time (h)	Temperature (˚C)	pH	Soluble protein	FAN	Peptide content	Soluble pentosan	Reducing sugars
1	8	20	6,03	40,43	241,70	11,69	0,60	24,19
2	24	20	4,39	41,26	338,17	14,18	0,64	10,75
3	8	35	5,58	43,94	305,49	13,91	0,54	22,35
4	24	35	4,01	53,88	474,84	24,44	0,61	2,97
5	8	27,5	6,47	42,30	316,59	12,70	0,51	26,71
6	24	27,5	4,05	44,26	424,56	16,66	0,69	2,88
7	16	20	5,4	39,78	299,00	11,60	0,59	25,12
8	16	35	3,81	44,09	421,29	16,80	0,60	3,47
9	16	27,5	4,05	39,10	323,24	13,48	0,67	6,22
10	16	27,5	4,31	37,82	349,32	13,19	0,60	6,03
11	16	27,5	4,08	39,09	358,47	13,28	0,69	5,18
12	16	27,5	4,07	39,33	367,97	13,32	0,63	5,26

Appendix 2. Bioprocessing conditions and measured values for responses for samples with strains and enzymes.

	Bioprocessing conditions			Response					
Sample	Time (h)	Temperature (˚C)	Enzyme dosage (nkat/g)	pH	Soluble protein	FAN	Peptide content	Soluble pentosan	Reducing sugars
1	8	20	5	6,32	35,73	211,71	11,22	2,04	32,01
2	24	20	5	4,37	38,69	336,80	14,56	2,16	14,42
3	8	35	5	5,87	38,92	299,91	13,84	2,18	34,44
4	24	35	5	4,24	48,08	499,73	21,97	2,61	5,97
5	8	20	500	6,4	40,34	277,50	14,56	8,30	50,88
6	24	20	500	4,48	42,16	402,36	18,47	9,31	48,01
7	8	35	500	5,73	43,53	348,97	18,45	7,41	60,33
8	24	35	500	4,25	49,98	653,73	26,83	9,27	47,18
9	8	27,5	50	6,16	37,00	255,99	13,29	3,64	41,71
10	24	27,5	50	4,02	43,16	412,89	18,32	4,08	15,07
11	16	20	50	5,81	37,36	276,81	13,47	3,88	39,46
12	16	35	50	4,32	38,93	392,41	17,96	4,34	22,28
13	16	27,5	5	4,64	38,65	332,57	14,42	1,64	15,63
14	16	27,5	500	4,26	41,07	400,53	18,80	8,58	51,87
15	16	27,5	50	4,32	37,75	323,70	14,29	3,86	28,04
16	16	27,5	50	4,48	33,70	220,97	10,43	3,51	16,11
17	16	27,5	50	4,51	38,11	318,72	14,26	3,76	26,16
18	16	27,5	50	4,27	37,43	327,00	14,31	3,41	25,43

Appendix 3 (1/3). Coefficient plots of response models for samples with strains.

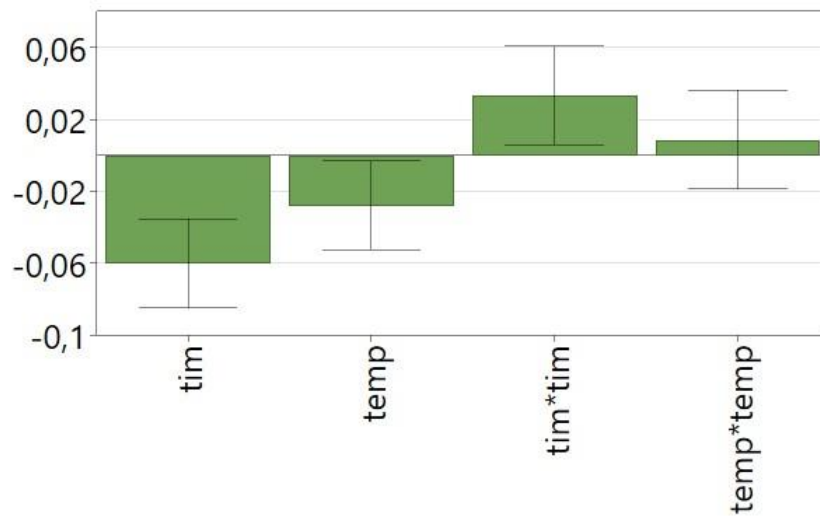

Figure 1. Coefficient plot of pH model for samples with strains.

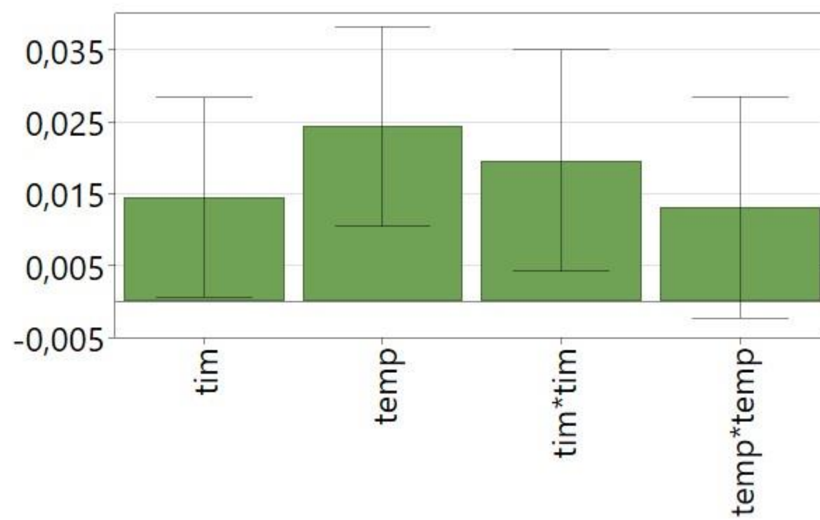

Figure 2. Coefficient plot of soluble protein model for samples with strains.

Appendix 3 (2/3). Coefficient plots of response models for samples with strains.

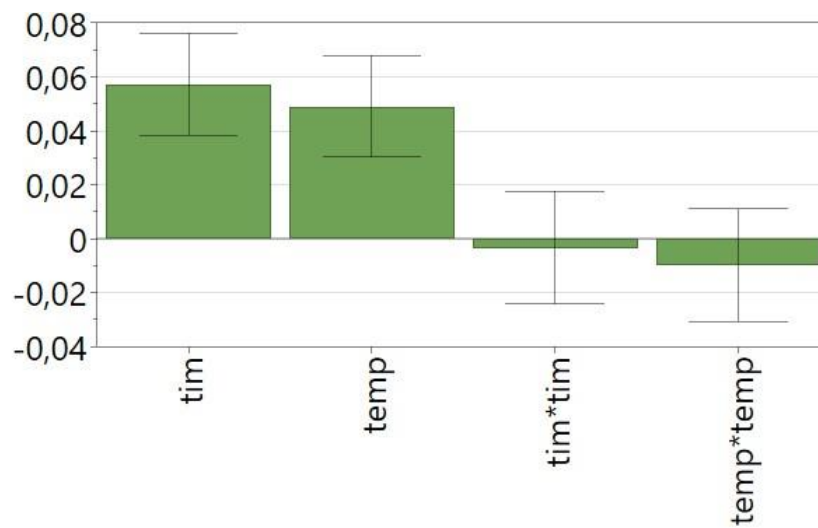

Figure 3. Coefficient plot of FAN model for samples with strains.

Figure 4. Coefficient plot of peptide content model for samples with strains.

Appendix 3 (3/3). Coefficient plots of response models for samples with strains.

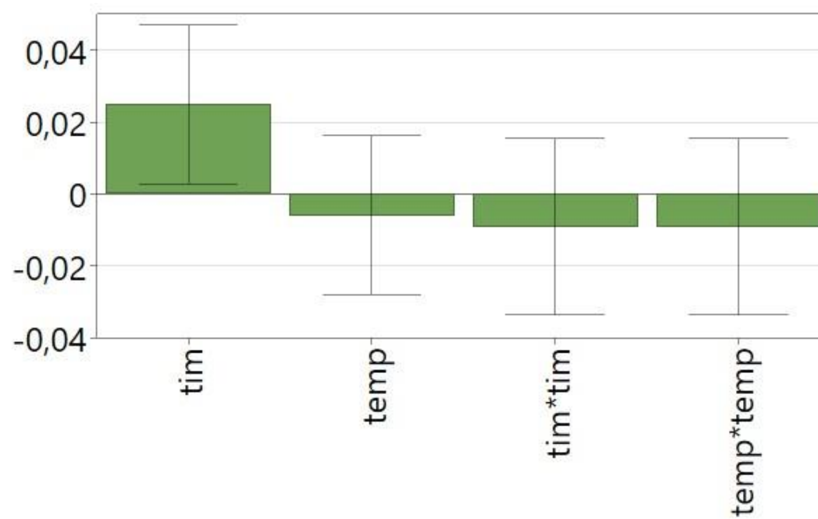

Figure 5. Coefficient plot of soluble pentosan model for samples with strains.

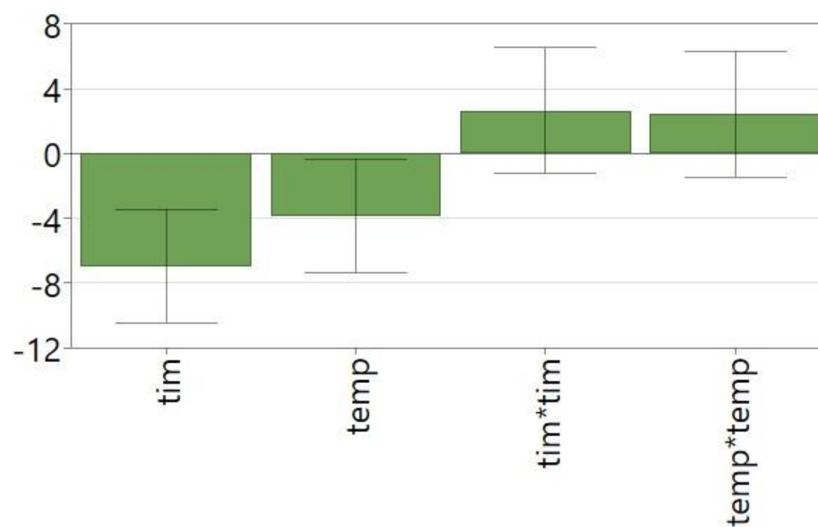

Figure 6. Coefficient plot of reducing sugars model for samples with strains.

Appendix 4 (1/3). Coefficient plots of response models for samples with strains and enzymes.

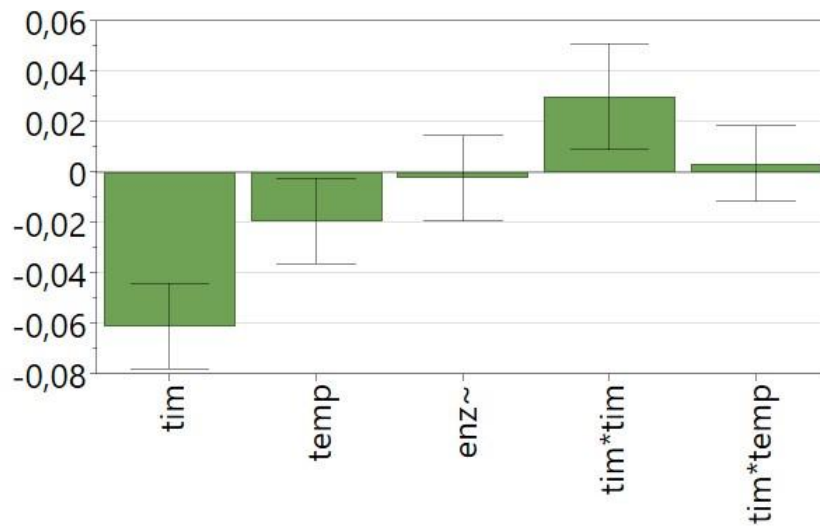

Figure 1. Coefficient plot of pH model for samples with strains and enzymes.

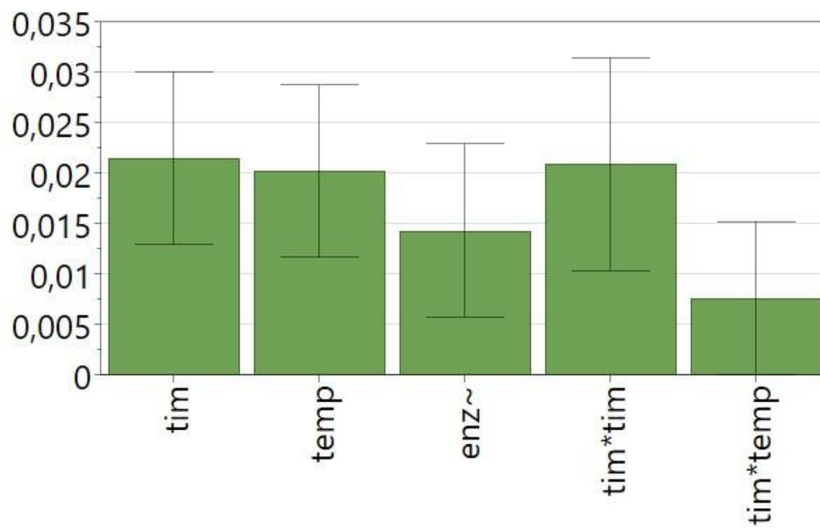

Figure 2. Coefficient plot of soluble protein model for samples with strains and enzymes.

Appendix 4 (2/3). Coefficient plots of response models for samples with strains and enzymes.

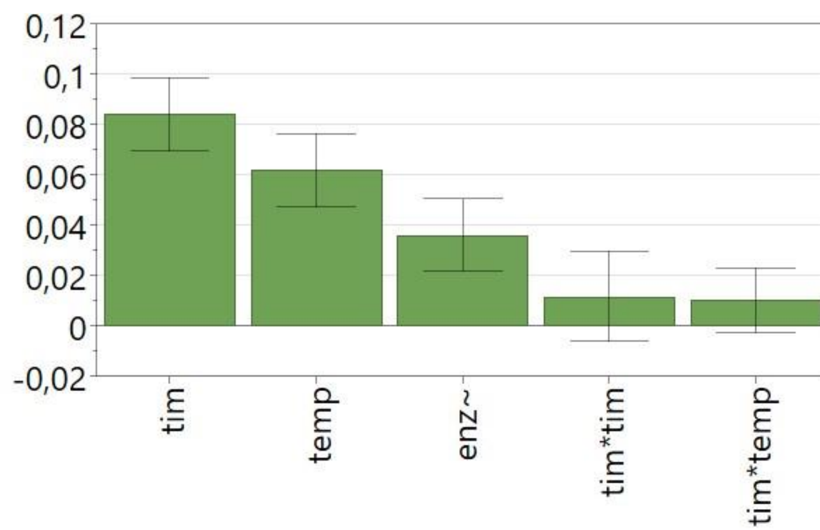

Figure 3. Coefficient plot of FAN model for samples with strains and enzymes.

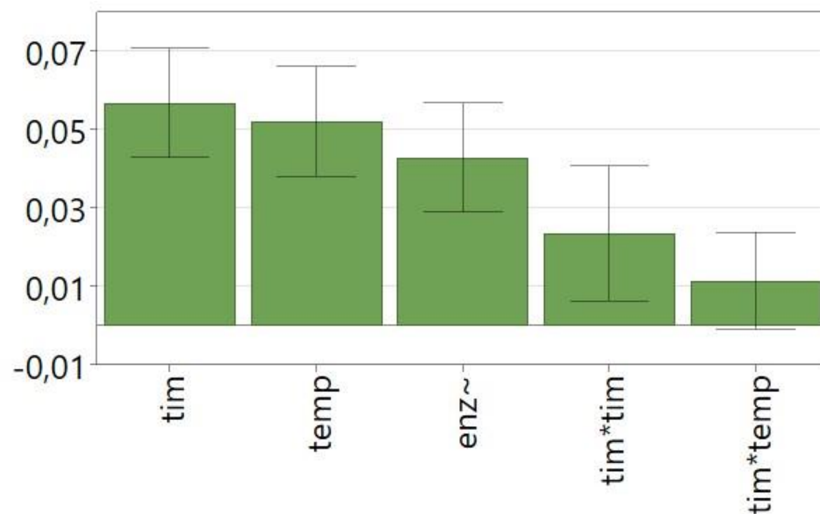

Figure 4. Coefficient plot of peptide content model for samples with strains and enzymes.

Appendix 4 (3/3). Coefficient plots of response models for samples with strains and enzymes.

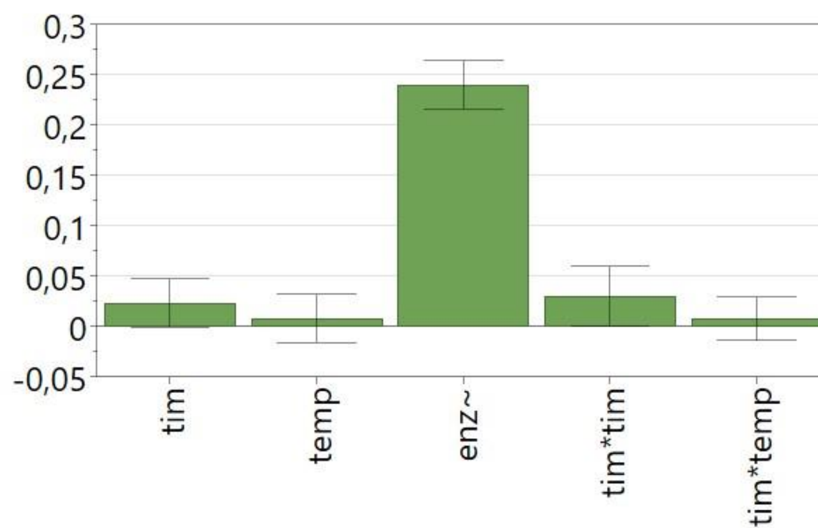

Figure 5. Coefficient plot of soluble pentosan model for samples with strains and enzymes.

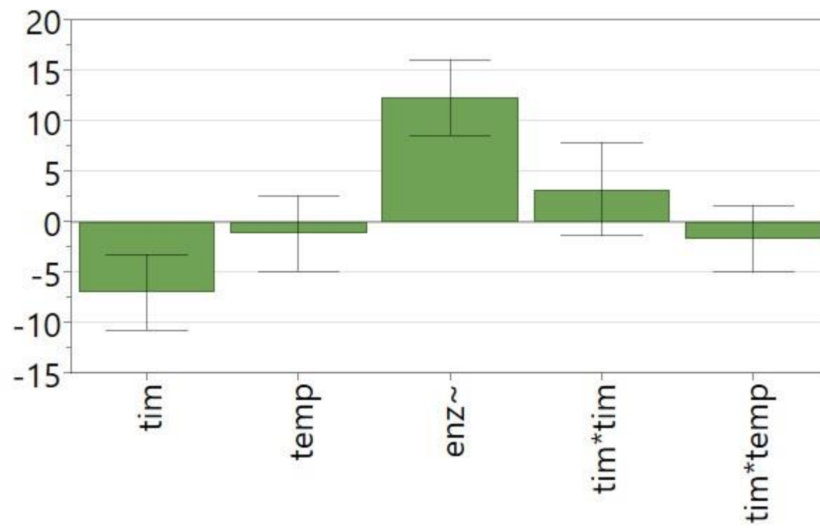

Figure 6. Coefficient plot of reducing sugars model for samples with strains and enzymes.